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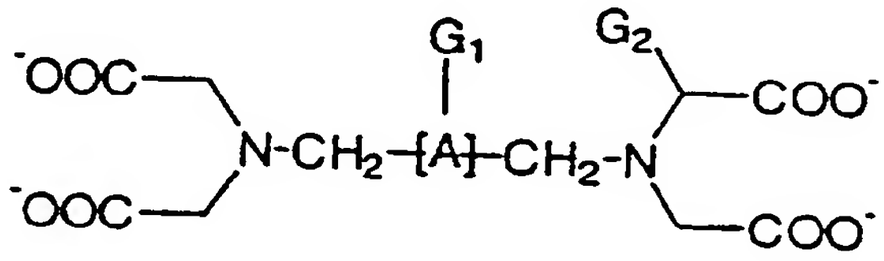
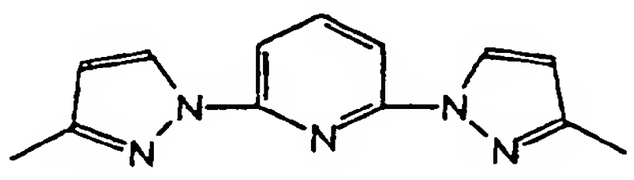
(54) Biospecific binding reactants labelled with luminescent lanthanide chelates and their use

(57) This invention relates to a detectable molecule comprising a biospecific binding reactant attached to a luminescent lanthanide chelate comprising a lanthanide ion and a chelating ligand of the formula



wherein
-A- is a bivalent aromatic structure selected from the group consisting of



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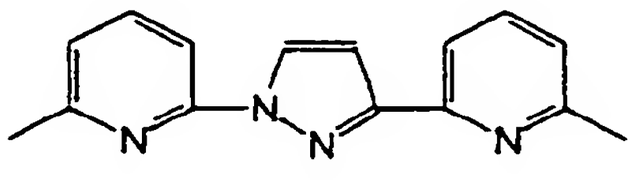

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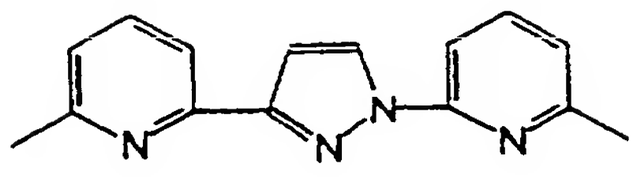


One of the two groups G_1 or G_2 is used for coupling the chelate to a biospecific binding reactant. The lanthanide ion is europium(III), terbium(III), dysprosium (III) or samarium (III).

Description

FIELD OF THE INVENTION

The present invention relates to detectables molecules comprising lanthanide chelates attached to a biospecific binding reactant and a use of the said detectable molecules in bioaffinity based binding assays. The invention further relates to novel luminescent lanthanide chelates useful in the preparation of said detectable molecules.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference.

In the specific binding assays, such as e.g. immunoassays, DNA hybridization assays, receptor-binding assays, and cellular binding assays, generally the analytes to be measured are present at very low concentration. Therefore various labelling compounds have been developed that allow the labelled reactant to be detected and quantitated at high sensitivity. In immunoassays and DNA hybridization assays time-resolved luminescence spectroscopy using lanthanide chelates is well known (e.g. I. Hemmilä, T. Ståhlberg, and P. Mottram (eds.), "Bioanalytical Applications of Labelling Technologies", Wallac, Turku, 1994). Stable photoluminescent (referred in the context of this simply as luminescent) lanthanide chelates also have other applications, e.g. fluorescence microscopy and cytometry. Therefore, a number of attempts have been made to develop new highly luminescent chelate labels suitable for those types of time-resolved fluorometric applications. These include e.g. stable chelates composed of derivatives of pyridines (US 4,920,195, US 4,801,722, US 4,761,481, PCT WO FI-91/00373, U.S. Pat. Appl. S.N. 08/135,525, Remuiñán, M.J., Román, H., Alonso, M.T. and Rodríguez-Ubis, J.C., 1993, J. Chem. Soc., Perkin Trans.2, 1099), bipyridines (US 5,216,134), terpyridines (US 4,859,777, US 5,202,423, US 5,324,825) or various phenolic compounds (US 4,670,572, US 4,794,191, Ital. Pat. 42508 A/89) at the energy mediating groups and polycarboxylic acids as chelating parts. In addition various dicarboxylate derivatives (US 5,032,677, US 5,055,578, US 4,772,563), macrocyclic cryptates (US 4,927,923, PCT WO 93/5049, EP-A493,745) and macrocyclic Schiff bases (EP-A369,000) have been patented. None of these terbium chelates do not fulfill all the required features to be used as labels for bioaffinity assays. These requirements are high thermodynamic and kinetic chelate stability, hydrophilicity, high absorptivity at a suitable excitation wavelength, appropriate triplet state to enable efficient energy transfer, high luminescence intensity, presence of a functional group, allowing the formation of a covalent linkage between the chelate and the target molecule, and the affinity and nonspecific binding properties of the used biomolecules must retain. In case of Tb^{III}, the energy gap between the excitation state of the donor ligand and the emitting level of the Tb^{III} ion should be high enough to prevent energy back transfer (Sabbatini, N., Mecati, A., Guardigli, M., Balzani, V., Lehn, J.-M., Zeissel, R. and Ungaro, R., 1991, J. Luminescence 48 & 49; 463-468). Moreover, with an exactly same ligand structure, none of those labels has enough high luminescence intensities with both Eu^{III} and Tb^{III}. In some applications, which contain various chromatographic separation steps, it would be preferable that the label structures differ from each other only with respect to the lanthanide used. Besides, the same key intermediates can be used in the preparations of several lanthanide labels, e.g. Eu^{III} and Tb^{III} labels.

SUMMARY OT THE INVENTION

According to the present invention, the problem of Tb^{III} chelate labels can be solved and the development of more luminescent Tb^{III} chelates is made possible. By preparing suitable ligand structures the energy back transfer from the donor ligand to the emitting level of the Tb^{III} ion i.e. the excited state deactivation route can be eliminated, and at the same time, all other important features of labels and labelled biomolecules can be retain.

One object of this invention is to provide a detectable molecule comprising a biospecific binding reactant attached to a luminescent lanthanide chelate according to this invention.

Another object of the present invention is to provide a luminescent lanthanide chelate.

Yet another object of the present invention is to provide the use of a detectable molecule comprising a biospecific binding reactant attached to the luminescent chelate according to this invention.

A further objective of this invention is to provide a chelating agent which has high luminescence intensity with several lanthanides, particularly with Tb^{III} and Eu^{III}, with an exactly same chelating structure.

DETAILED DESCRIPTION OF THE INVENTION

The aim of the present invention is to provide means to obtain improved lanthanide chelate labels to be used in specific bioaffinity based binding assays, such as immunoassays, DNA hybridization assays, receptor binding assays,

immunocytochemical or immunohistochemical assays utilizing fluorometric or time-resolved fluorometric determination of the specific luminescence.

The chelates of this invention have to combine several important features in the single label, such as:

1. high absorptivity at suitable wavelength (preferable over 300 nm).

2. efficient energy transfer from UV absorbing part (triplet sensitizer) to the chelated lanthanide(III) ion,

3. enough high the energy gap (preferable over 2000 cm^{-1}) between the triplet state of the donor ligand and the emitting level of the Tb^{III} ion ($^5D_4$ ca. 20500 cm^{-1}) to prevent energy back transfer,

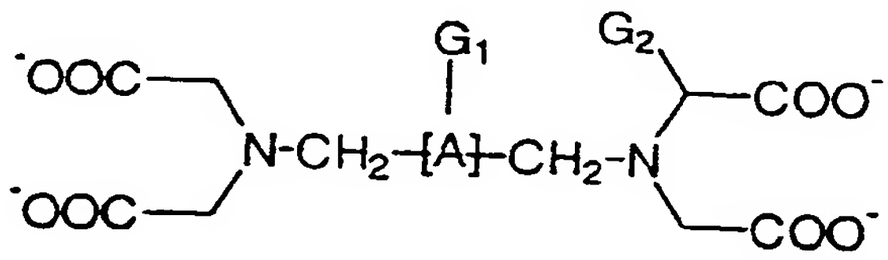
4. strongly chelating part to create a) the termodynamic stability required for storing the labelled reactants for extended periods of time, and b) high kinetic stability to allow the use of reactants in conditions where competing metal ions or chelating agents may be present,

5. chelating part forming as complete protection of the chelated ion as possible, preferable nine-dentate ligand.

6. functional group allowing efficient coupling of the chelate to be used binding reactant (e.g. antibody) without destroying its binding properties and decreasing the luminescence properties of the label.

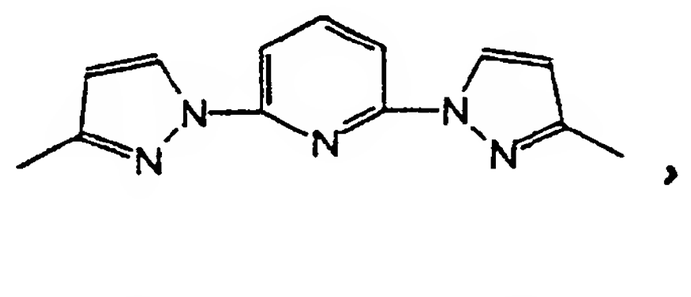
In addition, the chelate has to be highly hydrophilic and possess low nonspecific binding affinity to proteins or surfaces used in the analysis.

In one aspect therefore, the present invention provides a detectable molecule comprising a biospecific binding reactant attached to a luminescent lanthanide chelate comprising a lanthanide ion and a chelating ligand of the formula



wherein

a) -A- is a bivalent aromatic structure selected from the group consisting of



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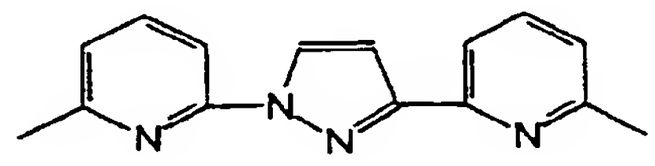

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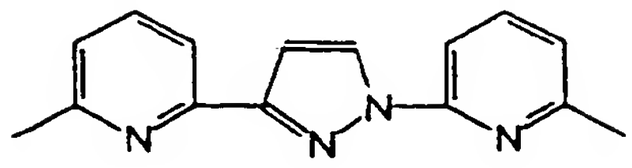
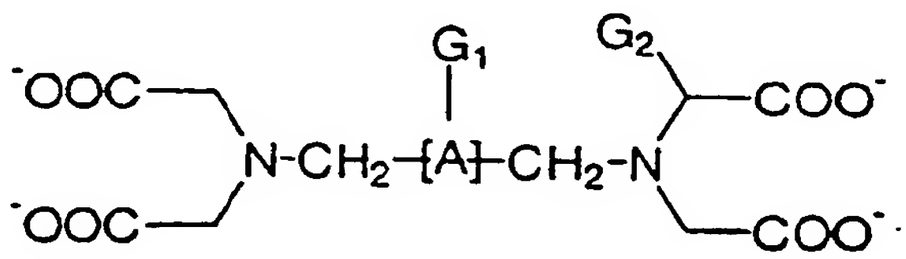


b) one of the groups G_1 or G_2 is selected from a group consisting of hydrogen, chloro, bromo, iodo, cyano, phenyl, alkyl and alkoxy, with the proviso that alkyls contain 1-6 carbon atoms; and the other group G_1 or G_2 is a bridge which is not participating in the chelating process and which is formed of one to four moieties, each moiety being selected from the group consisting of phenylene, alkylene containing 1-8 carbon atoms, ethynediyl (-C≡C-), ether (-O-), thioether (-S-) and amide (-CO-NH- and -NH-CO-);
c) one of the two groups G_1 or G_2 is used for coupling to a biospecific binding reactant wherein the group G_1 or G_2 selected for this purpose is coupled to biospecific binding reactant via thiourea (-NH-CS-NH-), aminoacetamide (-NH-CO-CH_2-NH-), amide (-NH-CO- and -CO-NH-), aliphatic thioether (-S-), disulfide (-S-S-) or 6-substituted-1,3,5-triazine-2,4-diamine; and
d) the lanthanide ion is europium(III), terbium(III), dysprosium (III) or samarium (III).

In another aspect, this invention relates to the use of a detectable molecule as defined above in biospecific binding assays.

The biospecific binding reactant is selected from a group consisting of an antibody, an antigen, a receptor ligand, a specific binding protein, a DNA- or RNA-probe.

In yet another aspect, this invention provides a luminescent lanthanide chelate comprising a lanthanide ion and a chelating ligand of the formula



wherein

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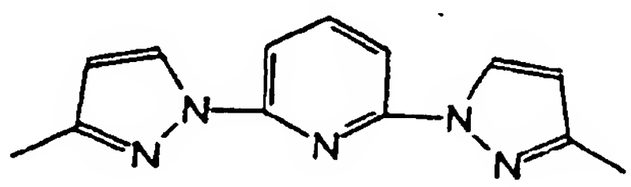

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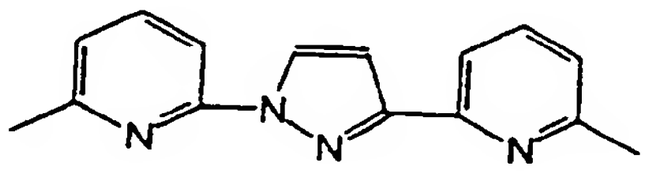

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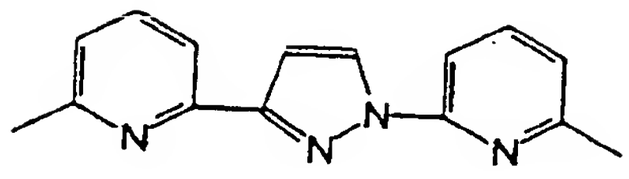


b) one of the groups G_1 or G_2 is selected from a group consisting of hydrogen, chloro, bromo, iodo, cyano, phenyl, alkyl and alkoxy, with the proviso that alkyls contain 1-6 carbon atoms; and the other group G_1 or G_2 is a substituent which is not participating in the chelating process and which is formed of one to four moieties, each moiety being selected from the group consisting of phenylene, alkylene containing 1-8 carbon atoms, ethynediyl (-C≡C-), ether (-O-), thioether (-S-) and amide (-CO-NH- and -NH-CO-) and additionally contains one moiety selected from a group containing hydroxy, nitro, amino, aminooxy, carboxyl, aldehyde or mercapto groups or an activated form made from them such as isocyanato, isothiocyanato, diazonium, bromoacetamido, iodoacetamido, reactive esters, pyridyl-2-dithio or 6-substituted 4-chloro-1,3,5-triazon-2-ylamino;
c) one of the two groups G_1 or G_2 is used for coupling to a biospecific binding reactant; and
d) the lanthanide ion is europium(III), terbium(III), dysprosium (III) or samarium (III).

The substituents in 6-substituted-1,3,5-triazine-2,4-diamine and 6-substituted 4-chloro-1,3,5-triazin-2-ylamino can be selected from the group consisting of hydrogen, halogen, alkoxy, aryloxy, amino, lower alkyl substituted amino or thioethers, preferable chloro, fluoro, ethoxy, 2-methoxyethoxy, 2-cyanoethoxy, 2,2,2-trifluoroethoxy, thiophenoxy or ethoxycarbonylthiomethoxy.

The term "luminescent" shall in this invention be understood to mean "photoluminescent" as already stated above.

The term "bivalent" shall be understood to mean a chemical group with two exactly known binding sites for two covalent bonds to two neigbour atoms.

According to a preferable embodiment of the invention the lanthanide ion is either europium (III) or terbium (III).

The invention is exemplified also with the following examples describing the dependence of the luminescence intensity on the triplet state of ligands and demonstrating importance of the triplet state of the labels on the luminescence intensity also after coupling the labels to biospecific binding molecules (e.g antibody). Also an example of chelate coupling to protein and standard curve of labelled antibody is given.

The structures and the synthetic routes employed in the experimental part are shown in reaction schemes 1 to 11. Scheme 1 illustrates the synthesis of compounds **1** to **4** exemplified by Examples 1 to 4. Scheme 2 illustrates the synthesis of compounds **5, 7** and **8** exemplified by Examples 5 to 7. Scheme 3 illustrates the synthesis of compound **9** exemplified by Example 8. Scheme 4 illustrates the synthesis of compounds **10** to **19** examplified by Examples 9 to 18. Scheme 5 illustrates the synthesis of compounds **20** to **27** starting from one of the compounds **18** or **19** as described in Examples 19-26. Scheme 6 illustrates the synthesis of compounds **28** to **31** starting from one of the compounds **22** or **23** and **4** according to Examples 27-30. Scheme 7 illustrates the synthesis of chelates **32** to **36** starting from one of the compounds **30** or **31** as described in Examples 31-35. Scheme 8 illustrates the synthesis of compounds **39** and **40** exemplified by Examples 36 and 37. Scheme 9 illustrates the synthesis of chelates **41-43** starting from compound 40 as described in Examples 38-40. Scheme 10 illustrates the synthesis of compounds **15, 25** and **44-46** starting from compound **13** as described in Examples 41-45. Scheme 11 illustrates the synthesis of chelates **47-49** starting from compound **46** according to Examples **46-48.**

Example 1.

The synthesis of copper(II) complex of bis[2-amino-6-(4-nitrobenzamido)hexanoic acid)] (1).

An aq. soln. (170 ml) of $CuSO_4 \times 5H_2O$ (12.5 g, 50 mmol) was added to a soln. of L-lysine x HCl (18.3 g, 100 mmol), NaOH (4.0 g, 100 mmol) and H_2O (170 ml). After stirring for 0.5 h at r.t., NaOH (4.0 g, 100 mmol) was added, and the reaction mixture was cooled at ice bath. A soln. of 4-nitrobenzoylchloride (37.1 g, 200 mmol) and 1,4-dioxane (250 ml) was added within 5 min, and the mixture was maintained basic with 1 M NaOH. After stirring for 0.5 h at ice bath, the basic mixture was stirred for 14 h at r.t., filtered, washed with cold H_2O and EtOH. Yield: 31.2 g (96 %). IR (KBr): 3412, 3279, 3151 (N-H), 1640, 1620, 1605 (C=O), 1528 (NO_2), 1349 (NO_2).

Example 2.

The synthesis of sodium salt of 2-amino-6-(4-nitrobenzamido)hexanoic acid) (**2**)

. A mixture of compound **1** (31.2 g, 47.8 mmol), disodium salt of (ethylenedinitrilo)tetra(acetic acid) x $2H_2O$ (21.9 g, 58.8 mmol) and H_2O (410 ml) was stirred for 3 h at 80°. The cold mixture was filtered, washed with cold H_2O and EtOH. Yield: 19.1 g (63 %). IR (KBr): 3422, 3334 (N-H), 1639, 1603 (C=O), 1522, 1352 (NO_2). ^{1}H-NMR (D_2O, (D_6) DMSO): 1.33-1.47 (*m*, 2 H); 1.55-1.64 (*m*, 2 H); 1.68-1.87 (*m*, 2 H); 3.33 (*t*, *J* = 7.0, 2 H); 3.42 (*t*, *J* = 5.8, 1 H); 8.01 (*d*, *J* = 8.8, 2 H); 8.34 (*d*, *J* = 8.8, 2 H).

Example 3.

The synthesis of methyl 2-amino-6-(4-nitrobenzamido)hexanoate (**3**).

$SOCl_2$ (12.4 ml, 0.17 mmol) was dropped slowly to cooled MeOH (250 ml). After stirring for 0.5 h at r.t., compound **2** (13.5 g, 42.5 mmol; twice co-evaporated with toluene (200 ml)) was added and the mixture refluxed for 3 h. After evaporation, the residue was dissolved to $CHCl_3$ (150 ml), neutralized with sat. $NaHCO_3$ and dried (Na_2SO_4). The product was purified by FC (silica gel, first $CHCl_3$, then 5 % MeOH): 8.54 g (65 %). IR (film): 3380, 3299 (N-H), 1732, 1650 (C=O), 1524, 1347 (NO_2), 1200 (C-O). ^{1}H-NMR ($CDCl_3$): 1.46-1.57 (*m*, 2 H); 1.58-1.89 (*m*, 4 H); 3.46-3.53 (*m*, 3 H); 3.73 (*s*, 3 H); 6.64 (*t*, *J* = 5.4, 1 H); 7.95 (*d*, *J* = 8.5, 2 H); 8.24 (*d*, *J* = 8.5, 2 H).

Example 4.

The synthesis of methyl 2-[N-(methoxycarbonylmethyl)amino]-6-(4-nitrobenzamido)hexanoate (**4**).

A mixture of compound 3 (4.27 g, 13.8 mmol), methyl bromoacetate (1.31 ml, 13.8 mmol), dry K_2CO_3 (9.53g, 69.0 mmol) and dry MeCN (80 ml) was refluxed for 4 h, filtered and evaporated. The product was purified by FC (silica gel, petroleum ether (40-60°)/EtOAc 2:5, then 0:1): 4.52 (86 %). IR (fim): 3333 (N-H), 1738, 1651 (C=O), 1526, 1348 (NO_2), 1207 (C-O). ^{1}H-NMR ($CDCl_3$): 1.42-1.60 (*m*, 2 H); 1.60-1.81 (*m*, 4 H); 2.18 (*bs*, 1 H); 3.31 (*dd*, *J* = 5.6, 7.1, 1 H); 3.35 (*d*, *J* = 17.4, 1 H); 3.44-3.50 (*m*, 2 H); 3.47 (*d*, *J* = 17.4); 3.71 (*s*, 3 H); 3.73 (*s*, 3 H); 7.00 (*t*, *J* = 5.4, 1 H); 7.99 (*d*, *J* = 8.5, 2 H); 8.25 (*d*, *J* = 8.5, 2 H).

Example 5.

The synthesis of methyl 2-amino-3-(4-nitrophenyl)propionate (**5**).

This compound (**5**) was synthesized from 4-nitro-L-phenylalanine x H_2O using a method analogous to the synthesis described in Example 3. Yield: 82 %. IR (KBr): 3384, 3314 (N-H), 1737 (C=O), 1518, 1347 (NO_2), 1200 (C-O). ^{1}H-NMR ($CDCl_3$): 2.98 (*dd*, *J* = 7.8, 13.7, 1 H); 3.18 (*dd*, *J* = 5.4, 13.7, 1 H); 3.69-3.81 (*m*, 1 H); 3.73 (*s*, 3 H); 7.40 (*d*, *J* = 8.8, 2 H); 8.17 (*d*, *J* = 8.8, 2 H).

Example 6.

The synthesis of methyl 2-[N-(methoxycarbonyl-methyl)amino] -3-(4-nitrophenyl)propionate (**7**).

This compound (**7**) was synthesized from **5** using a method analogous to the synthesis described in Example 4. FC (silica gel, first CH_2Cl_2, then 5 % MeOH/CH_2Cl_2). Yield: 36 %. IR (KBr): 3342 (N-H), 1740 (C=O), 1519,1347 (NO_2), 1203 (C-O). ^{1}N-NMR ($CDCl_3$): 3.04 (*dd*, *J* = 7.3, 13.7, 1 H); 3.13 (*dd*, *J* = 6.3, 13.7, 1 H); 3.36 (*d*, *J* = 17.3, 1 H); 3.42 (*d*, *J* = *17.3*, 1 H); 3.63 (*dd*, *J* = 6.3, 7.3, 1 H); 3. 69 (s, 3 H); 3.70 (*s*, 3 H); 7.39 (*d*, *J* = 8.8, 2 H); 8.16 (d, *J* = 8.8, 2 H).

Example 7.

The synthesis of methyl 2-[N-(methoxycarbonyl-methyl)amino] -6-[N-(benzyloxycarbonyl)amino]hexanoate (**8**).

This compound (**8**) was synthesized from **6** using a method analogous to the synthesis described in Example 4. FC (silica gel, petroleum ether (40-60°)/EtOAc first 5:3, then 2:5). Yield: 48 %. IR (KBr): 3343 (N-H), 1736 (C=O), 1244 (C-O). ^{1}N-NMR ($CDCl_3$): 1.35-1.90 (*m*, 6 H); 3.19 (*q*, *J*= 6.3, 2 H); 3.28 (*t*, *J* = 6.5, 1 H); 3.34 (*d*, *J* = 17.6, 1 H); 3.44 (*d*, *J* = 17.6, 1 H); 3.71 (*s*, 3 H); 3.72 (*s*, 3 H); 5.09 (*s*, 2 H); 7.28-7.40 (*m*, 5 H).

Example 8.

The synthesis of methyl N-(2-oxo-tetrahydrofur-3-yl)aminoacetate (**9**).

This compound (**9**) was synthesized from (±)-α-amino-γ-butyrolactone x HBr using a method analogous to the synthesis described in Example 4. FC (silica gel, $CHCl_3$). Yield: 31 %. IR (KBr): 3333 (N-H), 1770, 1740 (C=O), 1216, 1165 (C-O) ^{1}H-NMR ($CDCl_3$): 2.05-2.18 (*m*, 1 H); 2.47-2.57 (*m*, 1 H); 3.57 (*d*, *J* = 17.6, 1 H); 3.63 (*d*, *J* = 17.6, 1 H); 3.54 (*dd*, *J* = 8.1, 9.8, 1 H); 3.75 (*s*, 3 H); 4.21 (*dt*, *J* = 6.3, 9.8, 1 H); 4.42 (*dt*, *J* = 2.3, 8.8, 1 H).

Example 9

The synthesis of 3-(N,N-dimethylamino)-1-(2-pyridyl)-2-propen-1-one (**10**).

A mixture of N,N-dimethylformamide dimethyl acetal (6.65 g, 55.8 mmol) and 2-acetylpyridine (5.63 g, 46.5 mmol) was heated at 100°C for 4 hours. After evaporation, petroleum ether (40-60°, 50 ml) was added, the solid material was filtered, and washed with petroleum ether (40-60°). Yield: 6.76 g (83 %). UV (EtOH): 357, 245. IR (film): 1637 (C=O). ^{1}H-NMR ($CDCl_3$): 3.00 (*s*, 3 H); 3.18 (*s*, 3 H); 6.45 (*bd*, *J* = 12.6, 1 H); 7.36 (*ddd*, *J* = 1.2, 4.9, 7.8, 1 H); 7.80 (*dt*, *J* = 1.7, 7.8, 1 H); 7.91 (*d*, *J* = 12.6, 1 H); 8.15 (*bd*, *J* = 7.8, 1 H); 8.63 (*ddd*, *J* = 1.0, 1.7, 4.9, 1 H).

Example 10.

The synthesis of 3-(2-pyridyl)pyrazole (**11**).

A mixture of compound **10** (6.66 g, 37.8 mmol), NH_2NH_2 x H_2O (7.57 g, 151 mmol) and EtOH was stirred for 2 h at r.t., and was evaporated. Yield: 5.33 g (97 %). UV (EtOH): 281, 248. IR (film): 3156 (N-H), 1594 (arom). ^{1}H-NMR ($CDCl_3$): 6.81 (*d*, *J* = 1.9, 1 H); 7.23-7.26 (*m*, 1 H); 7.67 (*d*, *J* = 1.9, 1 H); 7.74-7.76 (*m*, 2 H); 8.67 (*dd*, *J* = 1.2, 4.9, 1 H).

Example 11.

The synthesis of 1,3-di(2-pyridyl)pyrazole hydrobromide (**12**).

A mixture of compound **11** (3.29 g, 22.7 mmol) and 2-bromopyridine (6.37 g, 40.3 mmol) was heated for 20 h at 185°C. The mixture was triturated with acetone (50 ml) and filtered. The product was purified with FC (silica gel, $CHCl_3$): 6.10 g (89 %). UV (EtOH): 296, 220 (sh). IR (KBr): . ^{1}H-NMR ($CDCl_3$): 17.32 (*ddd*, *J* = 0.9, 4.9, 7.3, 1 H); 7.88 (*ddd*, *J* = 1.5, 6.1, 7.3, 1 H); 7.94 (*dt*, *J* = 1.8, 8.1, 1 H); 7.97 (*d*, *J* = 2.8, 1 H); 8.30 (*d*, *J* = 8.1, 1 H); 8.46-8.50 (*m*, 2 H); 8.54 (*bd*, *J* = 8.1, 1 H); 8.73 (*d*, *J* = 2.8, 1 H); 9.00 (*bd*, *J* = 4.9, 1 H). ^{13}C-NMR ($CDCl_3$): 106.6, 112.6, 120.4, 121.5, 122.9, 128.4, 136.6, 138.6, 148.0, 149.5, 151.5, 151.8, 153.9.

Example 12.

The synthesis of 1,3-di(2-pyridyl)pyrazole (**13**).

A mixture of compound **11** (22.2 g, 153 mmol) and 2-bromopyridine (42.3 g, 268 mmol) was heated for 20 h at 190°C. The cooled mixture was dissolved in hot H_2O (200 ml) and the pH was adjusted to basic with solid Na_2CO_3. After cooling, the precipitate was filtered and washed with cold H_2O. Yield: 32.9 g (97 %). UV (EtOH): 294, 283, 224. IR (KBr): 1593, 1578 (arom). ^{1}H-NMR ($CDCl_3$): 7.14 (*d*, *J* = 2.6, 1 H); 7.21 (*ddd*, *J* = 1.0, 4.9, 7.3, 1 H); 7.27 (*ddd*, *J* = 1.1, 4.9, 7.3, 1 H); 7.79 (*dt*, *J* = 1.7, 7.3, 1 H); 7.85 (*dt*, *J* = 1.7, 7.3, 1 H); 8.12-8.16 (*m*, 2 H); 8.44 (*dd*, *J* = 1.7, 4.9, 1 H); 8.64 (*d*, *J* = 2.6, 1 H); 8.68 (*bd*, *J* = 4.9, 1 H).

Example 13.

The synthesis of 1,3-di(2-pyridyl)pyrazole N,N"-dioxide (**14**).

The 3-chloroperbenzoic acid (251 g, 50-55 %, ~730 mmol) was added in 7 portions to a solution of compound **13** (10.0 g, 45.0 mmol) in CH_2Cl_2 (1700 ml), and the mixture was stirred for 18 days at r.t.. H_2O (1400 ml) was added and the pH was adjusted to 10 with solid Na_2CO_3. The phases were separated and the water phase was extracted with $CHCl_3$/EtOH (3:1, 5 x 400 ml, 6 x 200 ml). The combined organic phase was dried with Na_2SO_4 and evaporated to dryness The product was purified with FC (silica gel, MeOH /$CHCl_3$ 1:9): 7.25 g (63 %). UV (EtOH): 307, 278, 257,

220. IR (film): 1228 (N→O). ^{1}H-NMR ($CDCl_3$): 7.23-7.27 (*m*, 1 H); 7.27-7.31 (*m*, 1 H); 7.36-7.41 (*m*, 1 H); 7.44-7.48 (*m*, 1 H); 7.84 (*d*, *J* = 2.7, 1 H); 8.20 (*dd*, *J* = 2.0, 8.3, 1 H); 8.29 (*dd*, *J* = 2.0, 8.1, 1 H); 8.39-8.43 (*m*, 2 H); 9.42 (*d*, *J* = 2.7, 1 H). ^{13}C-NMR ($CDCl_3$): 110.7, 120.3, 122.6, 124.9, 125.6, 126.2, 127.3, 128.0, 129.6, 130.0, 133.8, 140.8, 141.0.

Example 14.

The synthesis of 4-bromo-1,3-di(2-pyridyl)py-razole N,N'-dioxide (**15**).

This compound (**15**) was synthesized from **12** using a method analogous to the synthesis described in Example 13. Yield: 30 %. UV (EtOH): 295 (sh), 278, 259, 240 (sh). IR (film): 1256 (N→O). ^{1}H-NMR ($CDCl_3$): 7.23-7.27 (*m*, 1 H); 7.32-7.43 (*m*, 3 H); 7.52-7.55 (*m*, 1 H); 8.08-8.10 (*m*, 1 H); 8.34-8.36 (*m*, 1 H); 8.39-8.40 (*m*, 1 H); 9.48 (*s*, 1 H).

Example 15.

The synthesis of 1,3-bis(6-cyano-2-pyridyl)-pyrazole (**16**).

A mixture of compound **14** (7.20 g, 28.3 mmol), CH_2Cl_2 (250 ml) and Me_3SiCN (28.08 g, 283 mmol) was stirred at r.t. for 15 min. Benzoyl chloride (15.88 g, 113 mmol) was added and stirring was continued overnight. After evaporation to half a volume, 10 % K_2CO_3 (350 ml) was added, the mixture was stirred for 2 h and filtered. Yield: 5.59 g (73 %). UV (EtOH): 279 (sh), 275. IR (KBr): 2238 (C≡N), 1592, 1575 (arom). ^{1}H-NMR ((D6)-DMSO): 7.25 (*d*, *J* = 2.7, 1 H); 8.08 (*d*, *J* = 7.9, 2 H); 8.21 (*t*, *J* = 7.9, 1 H); 8.31 (*t*, *J* = 7.9, 1 H); 8.40 (*d*, *J* = 7.9, 1 H); 8.44 (*d*, *J* = 7.9, 1 H); 8.80 (*d*, *J* = 2.7, 1 H).

Example 16.

The synthesis of 4-bromo-1,3-bis(6-cyano-2-pyridyl)pyrazole (**17**).

This compound (**17**) was synthesized from **15** using a method analogous to the synthesis described in Example 15. Yield: 56 %. IR (KBr): 2237 (C≡N), 1590, 1574 (arom). ^{1}H-NMR ($CDCl_3$): 7.67 (*d*, *J* = 8.0, 1 H); 7.74 (*d*, *J* = 8.0, 1 H); 7.96 (*t*, *J* = 8.0, 1 H); 8.03 (*t*, *J* = 8.0, 1 H); 8.29 (*d*, *J* = 8.0, 1 H); 8.34 (*d*, *J* = 8.0, 1 H); 8.74 (*s*, 1 H).

Example 17.

The synthesis of dimethyl 1,3-di(2-pyridyl)pyrazole-6,6'-dicarboxylate (**18**).

A mixture of compound **16** (1.99 g, 7.3 mmol), sat. CH_3COOH (31 ml) and conc. H_2SO_4 (31 ml) was refluxed for 2 h, poured into ice H_2O (400 ml), filtered, treated with acetone, and the precipitate removed by centrifugation. $SOCl_2$ (1.8 ml, 24.3 mmol) was dropped slowly to cooled MeOH (130 ml). After stirring for 15 min at r.t., the above precipitate was added, and the mixture was refluxed for 4.5 h. After evaporation to a half volume, $CHCl_3$ (100 ml) was added, the mixture was neutralized with sat. $NaHCO_3$. The aq. phase was extracted with $CHCl_3$ (2 x 50 ml), the org. phase dried with Na_2SO_4, and evaporated. Yield: 1.77 g (66 %). UV (EtOH): 293, 220 (sh). IR (KBr): 1741 (C=O), 1588 (arom), 1234, 1141 (C-O). ^{1}H-NMR ($CDCl_3$): 4.03 (*s*, 3 H); 4.04 (*s*, 3 H); 7.28 (*d*, *J*=2.7, 1 H); 7.94 (*t*, *J* = 7.8, 1 H); 8.00 (*t*, *J* = 7.6, 1 H); 8.04 (*dd*, *J* = 1.5, 7.6, 1 H); 8.11 (*dd*, *J* = 1.0, 7.8, 1 H); 8.32 (*dd*, *J* = 1.0, 7.8, 1 H); 8.34 (*dd*, *J* = 1.5, 7.6, 1 H); 8.79 (*d*, *J* = 2.7, 1 H).

Example 18.

The synthesis of dimethyl 4-bromo-1,3-di(2-pyridyl)pyrazole-6,6'-dicarboxylate (**19**).

This compound (**19**) was synthesized from **17** using a method analogous to the synthesis described in Example 17. Yield: 57 %. UV (EtOH): 299. IR (film): 1739 (C=O), 1588 (arom), 1250, 1141 (C-O). ^{1}H-NMR ($CDCl_3$): 4.04 (*s*, 3 H); 4.05 (*s*, 3 H); 7.98 (*t*, *J* = 7.9, 1 H); 8.01 (*t*, *J* = 7.9, 1 H); 8.07 (*d*, *J* = 7.9, 1 H); 8.19 (*d*, *J* = 7.9, 1 H); 8.27 (*d*, *J* = 7.9, 1 H); 8.34 (*d*, *J* = 7.9, 1 H); 8.87 (*s*, 1 H).

Example 19.

The synthesis of 1,3-di(2-pyridyl)pyrazole-6,6'-dimethanol (**20**).

$NaBH_4$ (0.82 g, 21.6 mmol) was added to a suspension of compound **18** (1.76 g, 5.2 mmol) and abs. EtOH (35 ml). After stirring for 3 h at r.t., the mixture was refluxed for 1 h. The soln. was evaporated, sat. $NaHCO_3$ (40 ml) was added, the mixture brought to boiling. The cold mixture was filtered and washed with cold H_2O. Yield: 1.47 g (86 %). UV (EtOH): 299, 225(sh). IR (KBr): 1600, 1582 (arom). ^{1}H-NMR ((D6)-DMSO): 4.62 (*s*, 2 H); 4.64 (*s*, 2 H); 5.50 *(bs,* 1 H); 5.56 *(bs,* 1 H); 7.09 (*d*, *J* = 2.4, 1 H); 7.46 *(d, J* = 7.8, 1 H); 7.47 *(d, J* = 7.8, 1 H), 7.90 *(d, J* = 7.8, 1 H); 7.91 (*t*, *J* = 7.8, 1 H); 7.98 (*d*, *J* = 7.8, 1 H); 8.04 (*t*, *J* = 7.8, 1 H); 8.68 (*d*, *J* = 2.4, 1 H).

Example 20.

The synthesis of 4-bromo-1,3-di(2-pyridyl)-pyrazole-6,6'-dimethanol (**21**).

This compound (**21**) was synthesized from **19** using a method analogous to the synthesis described in Example 19. UV (EtOH): 301. IR (KBr): 1600, 1575 (arom). ^{1}H-NMR ($CDCl_3$): 3.05 (*t*, *J* = 5.1, 1 H); 4.48 (*t*, *J* = 5.1, 1 H); 4.82 *(d, J* = 5.1, 1 H); 7.20 *(d, J* = 7.6, 1 H); 7.24 *(d, J* = 7.6, 1 H); 7.80 (*t*, *J* = 7.6, 1 H); 7.88 (*t*, *J* = 7.6, 1 H); 7.99 *(d, J* = 7.6, 1 H); 8.04 *(d, J* = 7.6, 1 H); 8.71 (*s*, 1 H).

Example 21.

The synthesis of 1,3-bis(6-bromomethyl-2-pyridyl)pyrazole (**22**).

A mixture of dry DMF (20 ml) and PBr_3 (0.83 ml, 8.80 mmol) was stirred at r.t. for 15 min. Compound **20** (1.24 g, 4.40 mmol) was added in small portions, and the stirring was continued overnight. After neutralization with saturated $NaHCO_3$ solution, the precipitation was filtered, washed with cold H_2O and MeCN. The product was purified by FC (silica gel, CH_2Cl_2): 0.71 g (40 %). UV (EtOH): 302, 225 (sh). IR (KBr): 1598, 1577 (arom). ^{1}H-NMR ($CDCl_3$): 4.56 (*s*, 2 H); 4.63 (*s*, 2 H); 7.16 *(d, J* = 2.7, 1 H); 7.34 *(d, J* = 7.7, 1 H); 7.43 *(d, J* = 7.7, 1 H); 7.78 (*t*, *J* = 7.7, 1 H); 7.84 (*t*, *J* = 7.7, 1 H); 8.04 (*d*, *J* = 7.7, 2 H); 8.67 *(d, J* = 2.7, 1 H). ^{13}C-NMR ($CDCl_3$): 33.2, 34.1, 107.0, 111.8, 119.6, 121.0, 122.7, 128.6, 130.3, 137.6, 139.7, 151.5, 153.9, 155.5, 156.6.

Example 22.

The synthesis of 4-bromo-1,3-bis(6-bromomethyl-2-pyridyl)pyrazole (**23**).

This compound (**23**) was synthesized from **21** using a method analogous to the synthesis described in Example 21. Yield: 48 %. UV (EtOH): 302, 298, 230 (sh). IR (KBr): 1598, 1577 (arom). ^{1}H-NMR ($CDCl_3$): 4.54 (*s*, 2 H); 4.68 (*s*, 2 H); 7.37 *(dd, J* = 0.7, 7.6, 1 H); 7.52 *(dd, J* = 0.7, 7.6); 7.82 (*t*, *J* = 7.6, 1 H); 7.85 (*t*, *J* = 7.6, 1 H); 7.95 *(dd, J* = 0.7, 7.6, 1 H); 8.01 *(dd, J* = 0.7, 7.6, 1 H); 8.75 (*s*, 1 H).

Example 23.

The synthesis of tetra(*tert*-butyl) 2,2',2",2"'-{[6,6'-(pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl]bis(methylenenitrilo)} tetrakis(acetate) (**24**).

A mixture of compound **22** (15 mg, 37 µmol), di(*tert*-butyl) iminobis(acetate) (18 mg, 74 µmol), dry K_2CO_3 (50 mg, 370 µmol) and dry MeCN (300 µl) was refluxed 4.5 h. After evaporation, the residue was taken into $CHCl_3$ (10 ml), washed with H_2O (2 x 5 ml) and dried Na_2SO_4. Yield: 27 mg (100 %). UV (EtOH): 301, 215 (sh). IR (film): 1734 (C=O), 1143 (C-O). ^{1}H-NMR ($CDCl_3$): 1.47 (*s*, 36 H); 3.53 (*s*, 4 H); 3.57 (*s*, 4 H); 4.07 (*s*, 2 H); 4.15 (*s*, 2 H); 7.12 *(d, J* = 2.4, 1 H); 7.52 *(d, J* = 7.7, 1 H); 7.61 *(d, J* = 7.7, 1 H); 7.76 (*t*, *J* = 7.7, 1 H); 7.82 (*t*, *J* = 7.7, 1 H); 7.98 (*d*, *J* = 7.7, 1 H); 8.01 *(d, J* = 7.7, 1 H); 8.63 *(d, J* = 2.4, 1 H).

Example 24.

The synthesis of tetra(*tert*-butyl) 2,2',2",2"'-{[6,6'-(4"-bromopyrazole-1",3"-diyl)bis-(pyridine)-2,2'-diyl]bis (methylenenitrilo)}tetrakis-(acetate) (**25**).

This compound (**25**) was synthesized from **23** using a method analogous to the synthesis described in Example 23. Yield: 79 %. UV (EtOH): 300, 225. IR (film): 1732 (C=O), 1144 (C-O). ^{1}H-NMR ($CDCl_3$): 1.47 (*s*, 18 H); 1.48 (*s*, 18 H); 3.53 (*s*, 4 H); 3.57 (*s*, 8 H); 4.06 (*s*, 2 H); 4.15 (*s*, 2 H); 7.54 (*d*, *J* = 7.6, 1 H); 7.72 (*d*, *J* = 8.0, 1 H); 7.79 (*t*, *J* = 7.6, 1 H); 7.82 (*t*, *J* = 8.0, 1 H); 7.91 (*d*, *J* = 7.6, 1 H); 7.95 (*d*, *J* = 8.0, 1 H); 8.70 (s, 1 H).

Example 25.

The synthesis of 2,2',2",2"'-{[6,6'-(pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl]bis(methylenenitrilo)}tetrakis(acetic acid) (**26**).

A solution of compound **24** (63 mg, 65 µmol) in CF_3COOH (1.2 ml) was stirred at r.t. for 2 h. After evaporation to dryness, the residue was triturated with Et_2O, filtered and washed with Et_2O. UV (H_2O): 302, 224. UV ([Eu^{III} (**26**)], H_2O): 330, 320, 287, 279, 250 (sh). IR (KBr): 1734 (C=O), 1204 (C-O). ^{1}H-NMR ((D6)-DMSO): 3.62 (*s*, 4 H); 3.67 (*s*, 4 H); 4.10 (*s*, 2 H); 4.16 (*s*, 2 H); 7.13 (*d*, *J* = 2.2, 1 H); 7.52-7.55 (*m*, 2 H); 7.93-7.95 (*m*, 2 H); 8.03-8.05 (*m*, 2 H); 8.68 (*d*, *J* = 2.2, 1 H).

Example 26.

The synthesis of 2,2,2",2"'-{[6,6-(4"-bromopyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl]bis(methylenenitrilo)}tetrakis (acetic acid) (**27**).

This compound (**27**) was synthesized from **25** using a method analogous to the synthesis described in Example 25. UV (H_2O): 301, 226. UV ([Eu^{III}(**27**)], H_2O): 335 (sh), 323, 287, 280, 250 (sh). IR (KBr): 1734 (C=O), 1202 (C-O). ^{1}H-NMR ((D6)-DMSO): 3.67 (*s*, 4 H); 3.73 (*s*, 4 H); 4.15 (*s*, 2 H); 4.18 (*s*, 2 H); 7.56 (*d*, *J* = 7.6, 1 H); 7.63 (*d*, *J* = 7.6, 1 H); 7.91-8.00 (*m*, 3 H); 8.07 (*t*, *J* = 7.6, 1 H); 8.86 (*s*, 1 H).

Example 27.

The synthesis of tetramethyl 2- and 2"-[4-(4-nitrobenzamido)but-1-yl]-2,2',2",2"'-{[6,6'-(pyrazole-1",3"-diyl)bis (pyridine)-2,2'-diyljbis(methyl-enenitrilo)}tetrakis(acetate) (**28**)

Compound **4** (190 mg, 0.5 mmol) was added in two portions within 2 h to a mixture of compound **22** (204 mg, 0.5 mmol), dry K_2CO_3 (207 mg, 1.5 mmol) and dry MeCN (10 ml). After refluxing for 4 h, dimethyl iminobis(acetate) (81 mg, 0.5 mmol) was added. The mixture was refluxed overnight, filtered, the filtrate evaporated, and the residue was dissolved in dry pyridine (4 ml). After an addition of acetic anhydride (189 mg, 2 mmol), the soln. was stirred for 4 h at r.t., evaporated and the residue co-evaporated twice with toluene. The product was purified by FC (silica gel, petroleum ether (40-60°)/EtOAC/Et_3N 5/1/1): 150 mg (38 %). IR (film): 3349, 1732, 1651 (C=O), 1530, 1348 (NO_2), 1204 (C-O). ^{1}H-NMR ($CDCl_3$): 1.55-1.75 (*m*, 4 + 4 H); 1.75-1.90 (*m*, 2 + 2 H); 3.43-3.51 (*m*, 2 + 2 H); 3.55-3.75 (*m*, 3 + 3 H); 3.61 (*s*, 3 H); 3.62 (*s*, 3 H); 3.69 (*s*, 4 H); 3.70 (*s*, 4 H); 3.71 (*s*, 6 H); 3.72 (*s*, 6 H); 3.73 (*s*, 3 + 3 H); 3.95 (*d*, *J* = 14.6, 1 H); 3.99 (*d*, *J* = 14.6, 1 H); 4.05 (*d*, *J* = 14.6, 1 H); 4.08 (*s*, 2 H); 4.09 (*d*, *J* = 14.6, 1 H); 4.13 (*s*, 2 H); 6.83 (*bs*, 1 H); 6.96 (*bs*, 1 H); 7.06 (*d*, *J* = 2.4, 1 H); 7.07 (*d*, *J* = 2.4, 1 H); 7.35 (*d*, *J* = 7.8, 1 H); 7.40 (*d*, *J*= 7.8, 1 H); 7.45 (*d*, *J*= 7.8, 1 H); 7.52 (*d*, *J* = 7.8, 1 H); 7.59 (*t*, *J* = 7.8, 1 H); 7.68 (*t*, *J* = 7.8, 1 H); 7.76 (*t*, *J* = 7.8, 1 H); 7.82 (*t*, *J* = 7.8, 1 H); 7.88-7.98 (*m*, 2 + 2 H); 7.90 (*d*, *J* = 8.9. 2 H); 7.92 (*d*, *J* = 8.9, 2 H); 8.15 (*d*, *J* = 8.9, 2 H); 8.18 (*d*, *J* = 8.9, 2 H); 8.56 (*d*, *J* = 2.4, 1 H); 8.57 (*d*, *J* = 2.4, 1 H). It was impossible to assign signals to certain isomers. The isomeric ratio was according to NMR 56:44.

Example 28.

The synthesis of tetramethyl 2- and 2"-[4-(4-nitrobenzamido)but-1-yl]-2,2',2",2'"-{[6,6-(4"-bromopyrazole-1",3"-diyl)bis (pyridine)-2,2'-diyl]bis-(methylenenitrilo)}tetrakis(acetate) (**29**)

This compound (**29**) was synthesized from **23** using a method analogous to the synthesis described in Example 27. Yield: 30 %. IR (film): 3317 (N-H), 1737, 1659 (C=O), 1525, 1347 ($NO_{2)}$, 1210 (C-O). ^{1}H-NMR $CDCl_3$: 1.55-1.70

(*m*, 4 + 4 H); 1.75-1.88 (*m*, 2 + 2 H); 3.43-3.50 (*m*, 2 + 2 H); 3.50-3.75 (*m*, 3 + 3 H); 3.63 (*s*, 3 H); 3.65 (*s*, 3 H); 3.68 (*s*, 4 H); 3.72 (*s*, 4 + 6 H); 3.73 (*s*, 6 H); 3.73 (*s*, 3 + 3 H); 3.95 (*d, J = 14.6,* 1 H); 4.05 (*d, J* = 14.6, 1 H); 4.08 (*s*, 2 H); 4.09 (*d, J* = 14.6, 1 H); 4.13 (*d, J = 14.6,* 1 H); 4.17 (*s*, 2 H); 6.82 (*bs*, 1H); 6.94 (*bs*, 1 H); 7.41 (*d, J*= 7.6, 1 H); 7.49 (*d, J* = 7.6, 1 H); 7.53 (*d, J* = 7.6, 1 H); 7.63 (*d, J*= 7.6, 1 H); 7.64 (*t, J*= 7.6, H); 7.70 (*t, J* = 7.6, 1 H); 7.80 (*t, J*= 7.6, 1 H); 7.83 (*t, J*= 7.6, 1 H); 7.88-7.98 (*m*, 2 + 2 H); 7.93 (*d, J* = 8.8, 2 H); 7.96 *(d, J* = 8.8, 2 H); 8.17 (*d, J* = 8.8, 2 H); 8.20 (*d, J*= 8.8, 2 H); 8.65 (*s*, 1 H); 8.66 (*s*, 1 H). It was impossible to assign signals to certain isomers. The isomeric ratio was according to NMR 51:49.

Example 29.

The synthesis of tetramethyl 2- and 2"-[4-(4-aminobenzamido)but-1-yl]-2,2',2",2"'-{[6,6'-(pyrazole-1",3"-diyl)bis (pyridine)-2,2'-diyl]bis(methyl-enenitrilo)}tetrakis(acetate) (**30**)

A mixture of compound **28** (100 mg, 12 mmol), 10 % Pd/C (25 mg) and MeOH (20 ml) was stirred under H_2 (3.4 atm) for 0.5 h. After filtration, the filtrate was evaporated, and the product purified by FC (silica gel, petroleum ether (40-60°)/EtOAC 2/5): 58 mg (60 %). IR (film): 3348, 3244 (N-H), 1732, 1633 (C=O), 1180 (C-O). ^{1}H-NMR ($CDCl_3$): 1.53-1.72 (*m*, 4 + 4 H); 1.75-1.88 (*m*, 2 + 2 H); 3.43-3.50 (*m*, 2 + 2 H); 3.50-3.76 (*m*, 3 + 3 H); 3.65 (*s*, 3 + 3 H); 3.69 (*s*, 4 H); 3.70 (*s*, 4 H); 3.72 (*s*, 6 H); 3.73 (*s*, 6 + 3 H); 3.74 (*s*, 3 H); 4.03 (*d, J* = 14.6, 1 + 1 H); 4.09 (*s*, 2 H); 4.10 (*d, J* = 14.6, 1 + 1 H); 4.20 (*s*, 2 H); 6.60 (*d, J*= 8.5, 2 H); 6.61 (*d, J* = 8.5, 2 H); 7.08 (*d, J* = 2.4, 1 + 1 H); 7.42 (*d, J*= 7.6, 1 H); 7.44 (*d, J*= 7.6, 1 H); 7.59 (*d, J*= 8.5, 2 H); 7.61 (*d, J*= 8.5, 2 H); 7.62 (*t, J*= 7.6, 1 H); 7.67 (*t, J* = 7.6, 1 H); 7.76 (*t, J*= 7.6, 1 H); 7.82 (*t, J* = 7.6, 1 H); 7.90 (*d, J* = 7.6, 1 H); 7.94 (*d, J* = 7.6, 1 H); 7.97 (*d, J* = 7.6, 1 H); 7.98 (*d, J* = 7.6, 1 H); 8.59 (*d, J* = 2.4, 1 + 1 H). It was impossible to assign signals to certain isomers.

Example 30.

The synthesis of tetramethyl 2- and 2"-[4-(4-aminobenzamido)but-1-yl]-2,2',2",2"'-{[6,6'-(4"-bromopyrazole-1",3"-diyl) bis(pyridine)-2,2'-diyl]bis-(methylenenitrilo)}tetrakis(acetate) (**31**)

This compound (**31**) was synthesized from **29** using a method analogous to the synthesis described in Example 29. Yield: 65 %. IR (film): 3360, 3267 (N-H), 1738, 1627 (C=O), 1209 (C-O). ^{1}H-NMR ($CDCl_3$): 1.50-1.70 (*m*, 4 H); 1.75-1.85 (*m*, 2 H); 3.38-3.43 (*m*, 2 H); 3.50-3.76 (*m*, 3 H); 3.57 (*s*, 3 H); 3.69 (*s*, 4 H); 3.72 (*s*, 3 H); 3.75 (*s*, 6 H); 3.97 (*d, J* = 14.6, 1 H); 4.08 (*d, J* = 14.6, 1 H); 4.09 (*s*, 2 H); 6.63 (*d, J* = 8.5, 2 H); 7.47 (*d, J* = 8.0, 1 H); 7.50 (*d, J* = 8.0, 1 H); 7.58 (*d, J* = 8.5, 2 H); 7.70 (*t, J* = 8.0, 1 H); 7.85 (*t, J* = 8.0, 1 H); 8.01 (*d, J* = 8.0, 1 H); 8.04 (*d, J* = 8.0, 1 H); 8.65 (*s*, 1 H). Only one isomer shown.

Example 31.

The synthesis of 2- and 2"-[4-(4-aminobenz-amido)but-1-yl]-2,2',2",2"'-{[6,6'-(pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl] bis(methylene-nitrilo)}tetrakis(acetato)terbium(III) (**32**)

A mixture of compound **30** (67 mg, 88 µmol) and 0.5 M KOH in EtOH (2.5 ml) was stirred for 1 h at r.t.. Some H_2O (0.58 ml) was added and the stirring was continued for 3 h. After evaporation, the residue was dissolved in H_2O (1.2 ml) and the pH was adjusted to 6.5 with 5 M HCl. An aq. soln. (0.6 ml) of $TbCl_3$ (36 mg, 97 µmol) was added within 15 min and the pH was maintained at 5.0-6.5 with solid $NaHCO_3$. After stirring for 1.5 h at r.t. the pH was adjusted to 8.5 with 1 M NaOH. The precipitate was removed by centrifugation, the filtrate triturated with acetone, and the solid material removed by centrifugation and washed with acetone. The solid material was dissolved in H_2O (1.0 ml), extracted with phenol, and the phenol phase was treated with H_2O (1.0 ml) and Et_2O (10 ml). The H_2O phase was washed with Et_2O (2 x 10 ml), treated with acetone, and the product removed by centrifugation and washed with acetone. Yield: 30 mg (40 %). UV (H_2O): 331, 320, 287, 279, 263. IR (KBr): 1617 (C=O), 1388 (C-O).

Example 32.

The synthesis of 2- and 2"-[4-(4-aminobenz-amido)but-1-yl]-2,2',2",2"'-{[6,6'-(4"-bromopyrazole-1",3"-diyl)bis (pyridine)-2,2'-diyl]bis(methyl-enenitrilo)}tetrakis(acetato)terbium(III) (**33**)

This compound (**33**) was synthesized from **31** using a method analogous to the synthesis described in Example 31. UV (H_2O): 328, 317, 285, 277, 267. IR (KBr): 1602 (C=O), 1407 (C-O).

Example 33.

The synthesis of 2- and 2°-[4-(4-isothiocyanatobenzamido)but-1-yl]-2,2',2",2"'-{[6,6'-(pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl]bis(methyl-enenitrilo)}tetrakis(acetato)terbium(III) (**34**)

An aq. (0.454 ml) soln. of compound **32** (14 mg, 16 µmol) was added within 15 min to a mixture of thiophosgene (5.0 µl, 64 µmol), $NaHCO_3$ (6.7 mg, 80 µmol) and $CHCl_3$ (0.454 ml). After stirring for 30 min, the aq. phase was washed with $CHCl_3$ (3 x 1 ml), acetone was added to aq. soln. and the product removed by centrifugation and washed with acetone. Yield: 13 mg (87 %). UV (H_2O): 330, 320, 287, 280, 263. IR (KBr): 2099 (S=C=N), 1613 (C=O), 1366 (C-O).

Example 34.

The synthesis of 2- and 2"-[4-(4-isothiocyanatobenzamido)but-1-yl]-2,2',2",2"'-{[6,6'-(4"-bromopyrazole-1",3"-diyl)bis (pyridine)-2,2'-diyl]bis(methylenenitrilo)}tetrakis(acetato)terbium(III) (**35**)

This compound (**35**) was synthesized from 33 using a method analogous to the synthesis described in Example 33. Yield: 82 %. UV (H_2O): 328, 318, 286, 280, 264 (sh). IR (KBr): 2105 (S=C=N), 1603 (C=O), 1404 (C-O).

Example 35.

The synthesis of 2- and 2"-{4-{4-[(4,6-Dichloro-1,3,5-triazin-2-yl)amino]benzamido}but-1-yl}-2,2',2",2"'-{[6,6'-(pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl]bis(methylenenitrilo)}tetrakis(acetato)-terbium (III) (**36**)

A mixture of 2,4,6-trichloro-1,3,5-triazine (45 mg, 0.25 mmol), acetone (0.25 ml) and H_2O (0.25 ml) was added to a soln. of compound **32** (21 mg, 25 µmol) and 0.1 M NaOAC (0.375 ml, pH 4.9). After stirring for 15 min, acetone was added to the mixture, and the precipitate removed by centrifugation and washed with acetone. UV (H_2O): 330, 318, 287, 279, 262. IR (KBr): 1609 (C=O), 1388 (C-O).

Example 36.

The synthesis of trimethyl ethyl 2-[4-(4-nitrobenzamido)but-1-yl]-2,2',2",2"'-{[1,1'-(pyridine-2",6"-diyl)bis(pyrazole)-3,3'-diyl]bis(meth-ylenenitrilo)}tetrakis(acetate) (**39**)

Compound **37** (EP 298939 B1) (198 mg, 0.5 mmol) was added in two portions to a soln. of compound **38** (Remuiñán, M.J., Román, H., Alonso, M.T. and Rodríguez-Ubis, J.C., 1993, J. Chem. Soc., Perkin Trans.2, 1099) (200 mg, 0.5 mmol), dry K_2CO_3 (207 mg, 1.5 mmol) and dry MeCN (25 ml). After refluxing for 9 h, a soln. of dimethyl iminobis (acetate) (94.5 mg, 0.5 mmol) and dry MeCN (4 ml) was added in two portions. After refluxing for 7 h, the mixture was filtered, the filtrate evaporated and the product purified by FC (silica gel, EtOAc/hexane 3:2): 52 %. Anal. calcd for $C_{40}H_{51}N_9S_{11}$: C 57.61; H 6.16; N 15.12. Found: C 57.87; H 6.00; N 14.87.

Example 37.

The synthesis of trimethyl ethyl 2-[4-(4-aminobenzamido)but-1-yl]-2,2',2",2"'-{[1,1'-(pyridine-2",6"-diyl)bis(pyrazole)-3,3'-diyl]bis(meth-ylenenitrilo)}tetrakis(acetate) (**40**)

This compound (**40**) was synthesized from **39** using a method analogous to the synthesis described in Example 29. Anal. calcd for $C_{40}H_{53}N_9O_9$: C 59.76; H 6.65; N 15.68. Found: C 60.02; H 6.63; N 15.45.

Example 38.

The synthesis of 2-[4-(4-aminobenzamido)but-1-yl]-2,2',2",2"'-{[1,1'-(pyridine-2",6"-diyl)bis(pyrazole)-3,3'-diyl]bis (methylenenitrilo)}tetra-kis(acetato)terbium(III) (**41**)

A mixture of compound **40** (270 mg, 0.36 mmol) and 0.5 M KOH in EtOH (16 ml) was stirred for 2 h at r.t.. Some H_2O (1.6 ml) was added to the reaction mixture and the stirring was continued for 2 h. After evaporation, the residue was dissolved in H_2O (6 ml) and the pH was adjusted to 6.5 with 5 M HCl. An aq. soln. of $TbCl_3$ (134 mg, 0.36 mmol) was slowly added and the pH was maintained at 5.0-6.5 with 2 M NaOH. After stirring for 1 h, the precipitate was removed by centrifugation and washed with acetone. Yield: 60 %. UV (H_2O): 309, 276, 270. IR (KBr): 1616 (C=O),

1400 (C-O). Anal. calcd for $C_{32}H_{33}N_9O_9TbK$: C 43.40; H 3.76; N 14.23. Found: C 42.98; H 4.18; N 13.96.

Example 39.

The synthesis of 2-[4-(4-isothiocyanatobenz-amido)but-1-yl]-2,2',2",2"'-{[1,1'-(pyridine-2' ',6' '-diyl)bis(pyrazole)-3,3'-diyl]bis(methylenenitrilo)}tetra-kis(acetato)terbium(III) (**42**)

This compound (**42**) was synthesized from **41** using a method analogous to the synthesis described in Example 33. UV (H_2O): 313 (sh), 293, 277, 270 (sh), 253 (sh), 228. IR (KBr): 2077 (S=C=N), 1617 (C=O), 1381 (C-O).

Example 40.

The synthesis of 2-{4-{4-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]benzamido}but-1-yl}-2,2',2",2"'-{[1,1'-(pyridine-2",6"-diyl)bis(pyrazole)-3,3'-diyl]bis(methylenenitrilo)}tetrakis(acetato)terbium(III) (**43**)

This compound (**43**) was synthesized from **41** using a method analogous to the synthesis described in Example 35. UV (H_2O): 307, 276, 269, 242, 243 (sh). IR (KBr): 1608 (C=O), 1394 (C-O).

Example 41.

The synthesis of 4-bromo-1,3-di(2-pyridyl)pyrazole (**44**).

NBS (26.7 g, 150 mmol) and dibenzoylperoxide (1.20 g, 4.95 mmol) was added in 3 portions during 3 d to a boiling soln. of compound **13** (11.1 g, 50.0 mmol) and CCl_4 (220 ml). After refluxing additional 24 h, the cold mixture was filtered and the filtrate washed with sat. $NaHCO_3$ (100 ml), H_2O (100ml), dried (Na_2SO_4) and evaporated. Yield: 14.8 g (98 %). IR (KBr): 1598, 1588 (arom). ^{1}H-NMR ($CDCl_3$): 7.23-7.26 (*m*, 1 H); 7.32-7.35 (*m*, 1 H); 7.80-7.87 (*m*, 2 H); 8.09 (*d*, *J* = 7.8, 1 H); 8.14 (*d*, *J* = 8.3, 1 H); 8.43 (*d*, *J* = 4.1, 1 H); 8.72 (*s*, 1 H); 8.80 (*d*, *J* = 3.4, 1 H). MS: 300 (100, M^+), 302 (98, $[M + 2]^+$).

Example 42.

The synthesis of 4-bromo-1,3-di(2-pyridyl)py-razole N,N"-dioxide (**15**).

This compound (**15**) was synthesized from **44** using a method analogous to the synthesis described in Example 13. Yield: 49 %.

Example 43.

The synthesis of tetra(*tert*-butyl) 2,2'2",2"'-{[6,6'-(4"-bromopyrazole-1",3"-diyl)bis-(pyridine)-2,2'-diyl]bis (methylenenitrilo)}tetrakis-(acetate) (**25**).

A suspension of compound **17** (3.79 g, 10.8 mmol) and dry THF (100 ml) was deaerated with N_2. BH_3.THF (1 M, 110 ml) was added within 10 min into the cold reaction mixture. After stirring for 2 h in ice bath and 3 h at r.t., excess BH_3 was distroyed by addition of MeOH, the mixture evaporated and the residue dissolved in MeOH saturated with dry HCl (200 ml). After stirring for 1 h, the mixture was evaporated, and the residue treated with THF (150 ml). The cooled mixture was filtered and the solid material washed with THF. A mixture of this material, $BrCH_2COOC(CH_3)_3$ (2.91 ml, 18.0 mmol), dry $(i\text{-}Pr)_2EtN$ (12.0 ml, 67.5 mmol) and dry MeCN (90 ml) was refluxed for 24 h. After evaporation, the residue was dissolved in $CHCl_3$ (100 ml), washed with H_2O (3 x 50 ml) and dried (Na_2SO_4). The product was purified by FC (silica gel, petroleum ether (40-60°)/ACOEt/Et_3N 10:1:1): 0.97 g (26 %).

Example 44.

The synthesis of tetra(*tert*-butyl) 2,2',2",2"'-{{6,6'-{4"-{(4-aminophenyl)ethynyl]pyrazole-1",3"-diyl}bis(pyridine)-2,2'-diyl}bis(methyle-nenitrilo)}tetrakis(acetate) (**45**).

A mixture of compound **26** (0.49 g, 0.59 mmol), 4-amino-phenylacetylene (83 mg, 0.71 mmol; Takalo, H, Kankare, J. and Hänninen, E., 1988, Acta Chem. Scand. B 42, 448), dry Et_3N (3 ml) and dry THF (3 ml) was deaerated with N_2. Bis(triphenylphosphine)palladium(II) chloride (8.5 mg, 12 µmol) and CuI (4.6 mg, 24 µmol) was added and the mixture

was stirred for 40 h at 55-60°. After evaporation, the residue was dissolved in $CHCl_3$ (20 ml), washed with H_2O (3 x 10 ml) and dried (Na_2SO_4). The product was purified by FC (silica gel, petroleum ether (40-60°)/AcOEt first 5:3 then 2:5): 0.12 g (24 %). IR (film): 3466, 3372 (N-H), 2215 (C≡C), 1737 (C=O), 1146 (C-O). ^1H NMR ($CDCl_3$): 1.45 (*s*, 18 H); 1.48 (*s*, 18 H); 3.54 (*s*, 4 H); 3.55 (*s*, 4 H); 4.06 (*s*, 2 H); 4.21 (*s*, 2 H); 6.66 (*d*, *J* = 8.4, 2 H); 7.35 (*d*, *J* = 8.4, 2 H); 7.53 (*d*, *J* = 7.3, 1 H); 7.76 (*d*, *J* = 7.3, 1 H); 7.80 (*t*, *J* = 7.3, 1 H); 7.82 (*t*, *J* = 7.3, 1 H); 8.03 (*d*, J = 7.3, 1 H); 8.18 (*d*, *J* = 7.3, 1 H); 8.83 (*s*, 1 H).

Example 45.

The synthesis of tetra(*tert*-butyl) 2,2',2",2'"-{{{6,6'-{4"-[2-(4-aminophenyl)ethyl]pyrazole-1",3"-diyl}bis(pyridine)-2,2'-diyl}bis(methyle-nenitrilo)}tetrakis(acetate) (**46**).

A mixture of compound **46** (115 mg, 0.135 mmol), 10 % Pd/C (20 mg) and MeOH (20 ml) was stirred under N_2 (0.69 MPa) for 2.5 h. After filtration, the filtrate was evaporated and the residue was purified by FC (silica gel, petroleum ether (40-60°)/AcOEt 5:3): 87 mg (75 %). IR (film): 3444, 3372 (N-H), 1733 (C=O), 1145 (C-O). ^1H NMR ($CDCl_3$): 1.44 (*s*, 18 H); 1.47 (*s*, 18 H); 2.84-2.89 (*m*, 2 H); 3.19-3.23 (*m*, 2 H); 3.52 (*s*, 4 H); 3.53 (*s*, 4 H); 4.05 (*s*, 2 H); 4.11 (*s*, 2H); 6.63 (*d*, *J* = 7.9, 2 H); 7.03 (*d*, *J* = 7.9, 2 H); 7.48 (*d*, *J* = 7.9, 1 H); 7.62 (*d*, *J* = 7.9, 1 H); 7.74 (*t*, *J* = 7.9, 1 H); 7.78 (*t*, *J* = 7.9, 1 H); 7.92 (*d*, *J* = 7.9, 1 H); 7.93 (*d*, *J* = 7.9, 1 H); 8.38 (*s*, 1 H).

Example 46.

The synthesis of 2,2',2",2'"-{{6,6'-{4"-[2-(4-aminophenyl)ethyl]pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl}bis (methylenenitrilo)}tetrakis-(acetato)terbium(III) (**47**).

First, the ester group hydrolysis of compound **45** using a method analogous to the synthesis described in Example 25 gave 2,2',2",2'"-{{6,6"-{4"-[2-(4-aminophenyl)ethyl]py-razole-1",3'-diyl}bis(pyridine)-2,2'-diyl}bis(methylene-nitrilo)} tetrakis(acetic acid). UV (H_2O): 335 (sh), 304, 226 (sh). UV([EU^{III} (**46**)], H_2O): 325, 289, 279 (sh), 238 (sh). IR (KBr): 1734, 1676, 1636 (C=O), 1376, 1202 (C-O). ^1H NMR ((D_6)-DMSO): 2.96 (*t*, *J* = 7.7, 2 H); 3.22 (*t*, *J* = 7.7, 2 H); 3.59 (*s*, 4 H); 3.60 (*s*, 4 H); 4.07 (*s*, 2 H); 4.11 (*s*, 2 H); 7.15 (*d*, *J* = 7.7, 2); 7.33 (*d*, *J* = 7.7, 2 H); 7.50 (*d*, *J* = 7.7, 1 H); 7.57 (*d*, *J* = 7.7, 1 H); 7.89 (*d*, *J* = 7.7, 1 H); 7.92 (*t*, *J* = 7.7, 1 H); 7.97 (*d*, *J* = 7.7, 1 H); 8.01 (*t*, *J* = 7.7, 1 H); 8.48 (*s*, 1 H). This tetraacid (43 mg, 40 µmol) was dissolved in H_2O (0.75 ml) and the pH was adjusted to 6.5 with solid $NaHCO_3$. An aq. soln. (0.25 ml) of $TbCl_3$ (16 mg, 44 µmol) was added within 15 min and the pH was maintained at 5-7. After stirring for 2 h, the pH was raised to 8.5-9.0 with 1 M NaOH, the precipitate removed by centrification, the filtrate treated with acetone, and the precipitate removed by centrification and washed with acetone. Yield: 27 mg (84 %). UV (H_2O): 338 (sh), 327, 290, 283 (sh), 239. IR (KBr): 1608 (C=O), 1396 (C-O).

Example 47.

The synthesis of 2,2',2",2'"-{{6,6'-{4"-[2-(4-isothiocyanatophenyl)ethyl]pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl}bis (methylenenitrilo)}tetrakis-(acetato)terbium(III) (**48**).

This compound (**48**) was synthesized from **47** using a method analogous to the synthesis described in Example 33. UV (H_2O): 337 (sh), 326, 289 (sh), 281, 270, 254. IR (KBr): 2115 (S=C=N), 1609 (C=O), 1397 (C-O).

Example 48.

The synthesis of 2,2',2",2'"-{{6,6'-{4"-{2-[(4,6-Dichloro-1,3,5-triazin-2-yl)amino]ethyl}pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl}bis(methylene-nitrilo)}tetrakis(acetato)terbium(III) (**49**).

This compound (**49**) was synthesized from **47** using a method analogous to the synthesis described in Example 35. UV (H_2O): 321, 290, 251. IR (KBr): 1608 (C=O), 1390 (C-O).

Example 49.

The luminescence measurements of compound **26** and **27** with Eu^{III} and Tb^{III}.

The luminescence parameters for the Eu^{III} and Tb^{III} chelates were measured in borate buffer, pH 8.5. The ligand

concentrations were kept at 10 μM, and the lanthanide-ion concentrations varied between 0.1 and 1 μM depending on the luminescence intensities. The measurements were standardized using 0.1 μM Eu^{III} in the *Wallac Delfia* enhancement soln. (molar absortivity 37600, quantum yield 70 % and luminescence yield 26320). The emission intensities of the lanthanide chelates were measured using the most intense emission line, at *ca.* 545 nm for Tb^{III} and 613 for Eu^{III}. The Tb^{III} luminescence was corrected for photomultiplier quantum-yield difference (1.39-fold at 545 nm as compared to the value at 613 nm). The phosphorescence spectra were measured in 5:4 mixtures of glyserol (purified) and water (quartz dist.) buffered by tris (hydroxymethyl)aminomethane and HCl (TRIS-HCl buffer). Concentration of Gd^{III} (added as perchlorate) was 10 μM and ligand 30 μM.

Table 1

The triplet state energy levels (E) of Gd^{III} chelates of compounds **26, 27** and TERPY, and the excitation maxima (λ_{exc}), luminescence decay times (τ) and luminescence yields ($\varepsilon \cdot \Phi$) of the Eu^{III} chelates of compounds **26, 27** and TERPY.

Compound	λ_{exc}[nm]	τ[μs]	$\varepsilon \cdot \Phi$	E[cm^{-1}]
Eu^{III}(**26**)	327	1000	1550	23000
Eu^{III}(**27**)	329	1020	1630	23100
Eu^{III}(TERPY)	334	1310	2100	22400

TERPY is 2,2',2",2"'-[(2,2':6',2"-terpyridine-6,6"-diyl)bis(methylenenitrilo)]tetrakis(acetic acid)

Table 2

The triplet state energy levels (E) of Gd^{III} chelates of compounds **26, 27** and TERPY, and the excitation maxima (λ_{exc}), luminescence decay times (τ) and luminescence yields ($\varepsilon \cdot \Phi$) of the Tb^{III} chelates of compounds **26, 27** and TERPY.

Compound	λ_{exc}[nm]	τ[μs]	$\varepsilon \cdot \Phi$	E[cm^{-1}]
Tb^{III}(**26**)	327	2810	8810	23000
Tb^{III}(**27**)	331	2550	8900	23100
Tb^{III}(TERPY)	333	1100	3800	22400

Example 50. Coupling of the chelates to Protein.

The activated chelates were coupled to a model protein (PSA-antibody, clone H50) by incubating the chelate with IgG (1 mg/ml) in carbonate buffer (500 μl, pH 9.8) overnight using a 30-fold molar reactant-to-protein ratio. After the coupling reaction, the protein was purified on a column of *Superdex 200 prep grade* by eluting with 50 mM *Tris*-HCl buffer (pH 7.75) containing 0.15 M NaCl and 0.05 % NaN_3 soln. The fractions corresponding labelled monomeric IgG were collected. The chelate concentrations in the protein fractions were measured from both the absorptions of the conjucated chelate at 330 nm and the total Tb^{III} ion concentration measured by the dissociative fluorescence enhancement system. The purified protein conjugate and the labelling ratio (chelates per protein) were quantitated by calculating the protein yield or by measuring the absor-bance at 280 nm and subtracting the absorption caused by the added chelate.

Example 51.

The luminescence measurements of chelate-labelled antibodies.

The luminescence measurements of chelate-labelled antibodies were measured similarly as in Example 49 using appropriate dilutions of the conjucated proteins analyzed above.

Table 3

The excitation maxima (λ_{exc}), luminescence decay times (τ) and luminescence yields ($\varepsilon \cdot \Phi$) of the Tb^{III} chelates 34, 42, 43 and TERPY in protein

Chelate	λ_{exc}[nm]	τ[μs]	$\varepsilon \cdot \Phi$
34	328	1350	3860

Table 3 (continued)

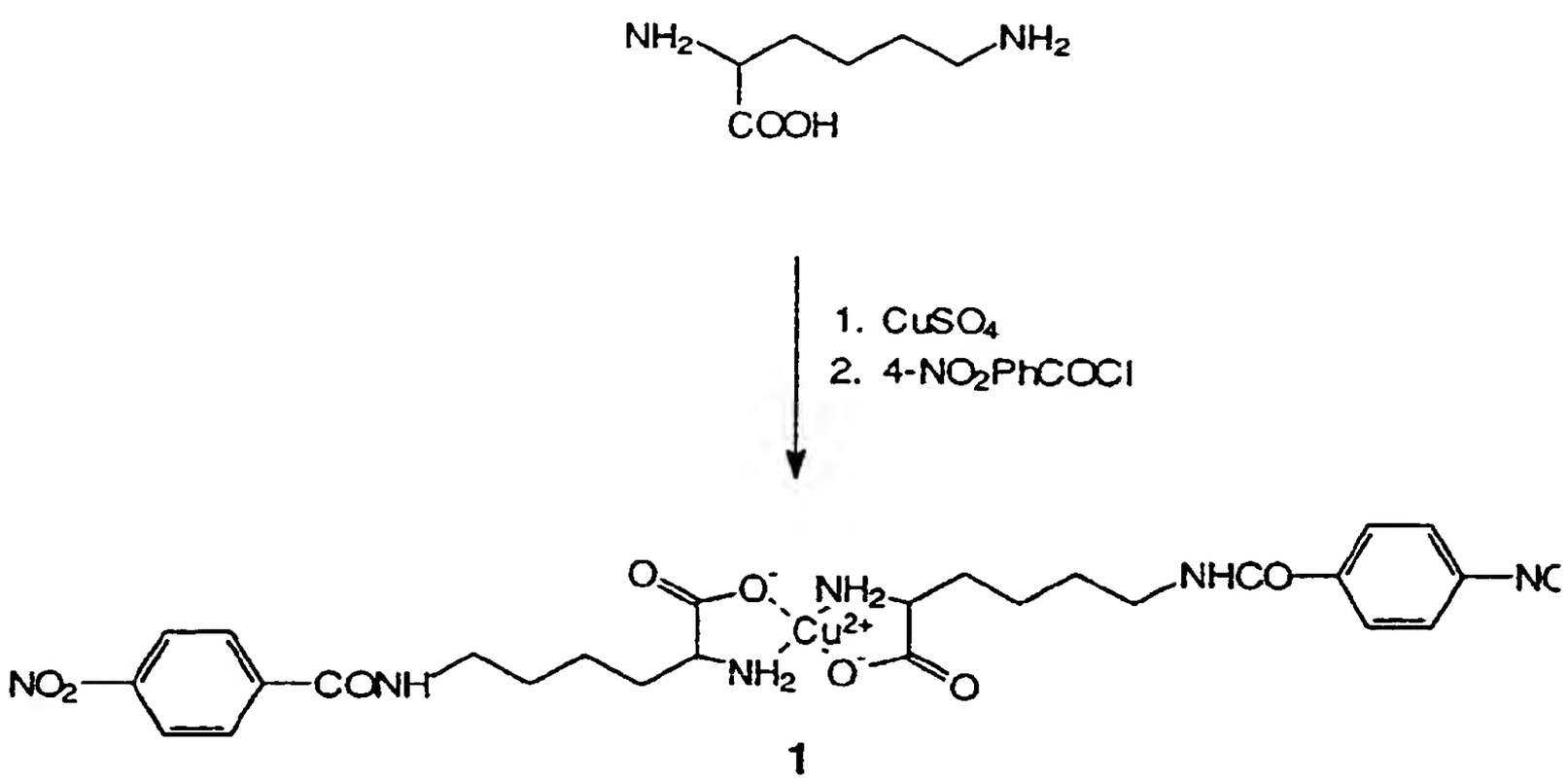
Chelate	λ_{exc}[nm]	τ[μs]	ε·Φ
42	315	2930	3770
43	310	2670	4050
Tb^{III}(TERPY) in protein	333	330	1170
Tb^{III}TERPY in protein: see Mukkala, V.-M., Takalo, H., Liitti, P. and Hemmilä, I., 1995, J. Alloys and Compounds 225, 507-510: compound 11 a.			

Example 52.

Standard curve

A PSA standard curve where a PSA-antibody labelled with chelate **42** is used as a label. The curve is shown in Figure 1.

It will be appreciated that the methods and compositions of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the skilled person that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.



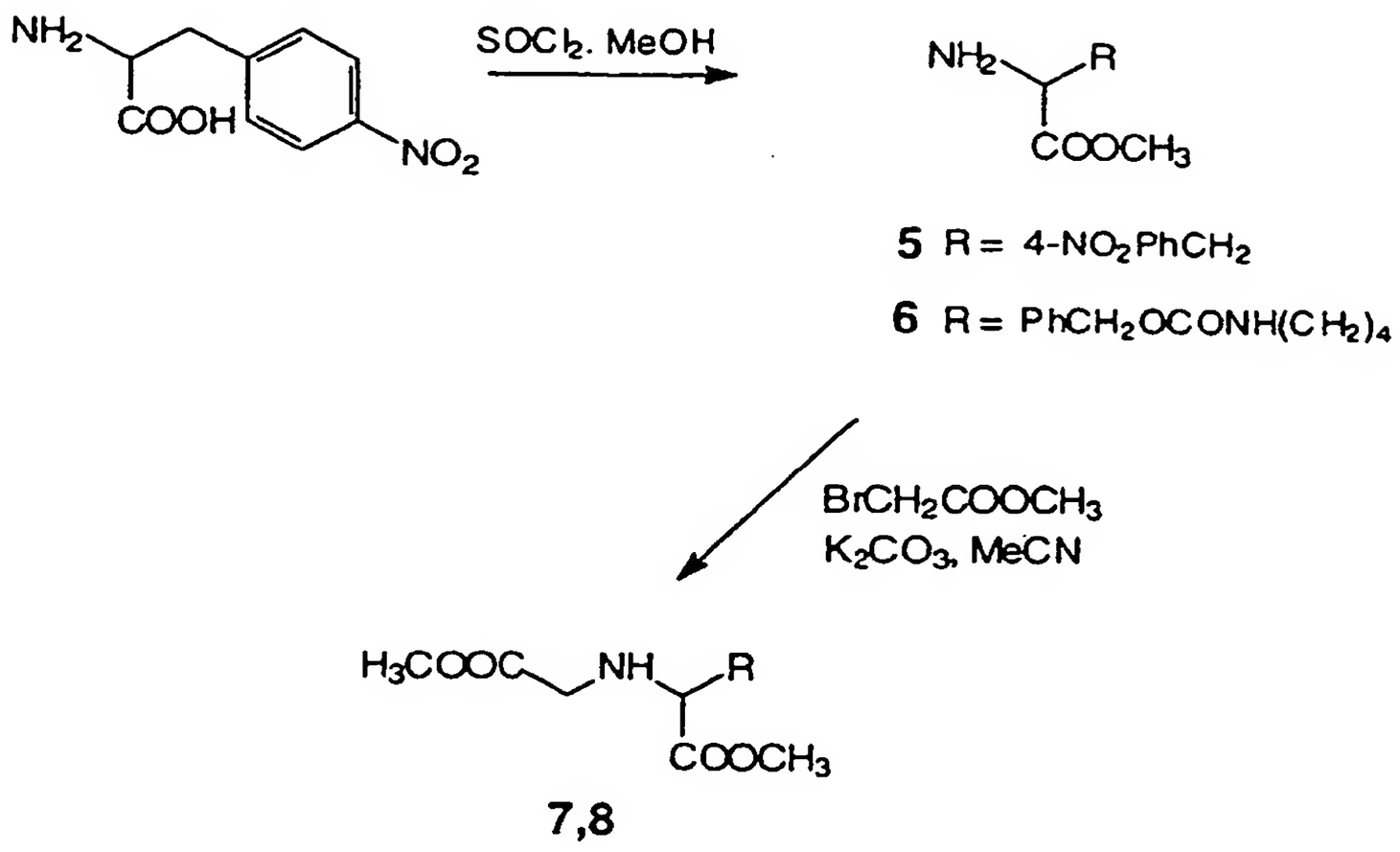
1

2

3

4

SCHEME 1.



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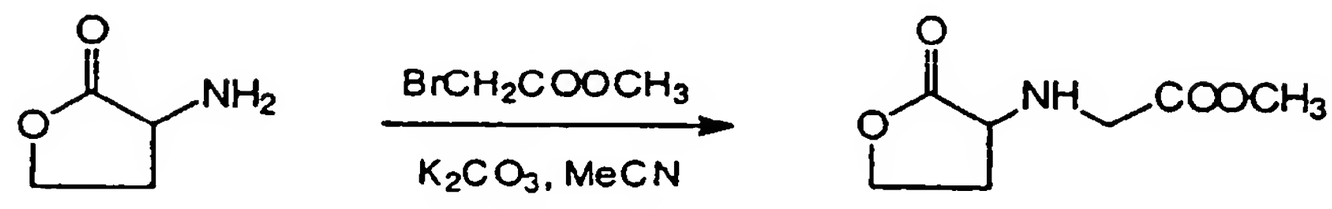

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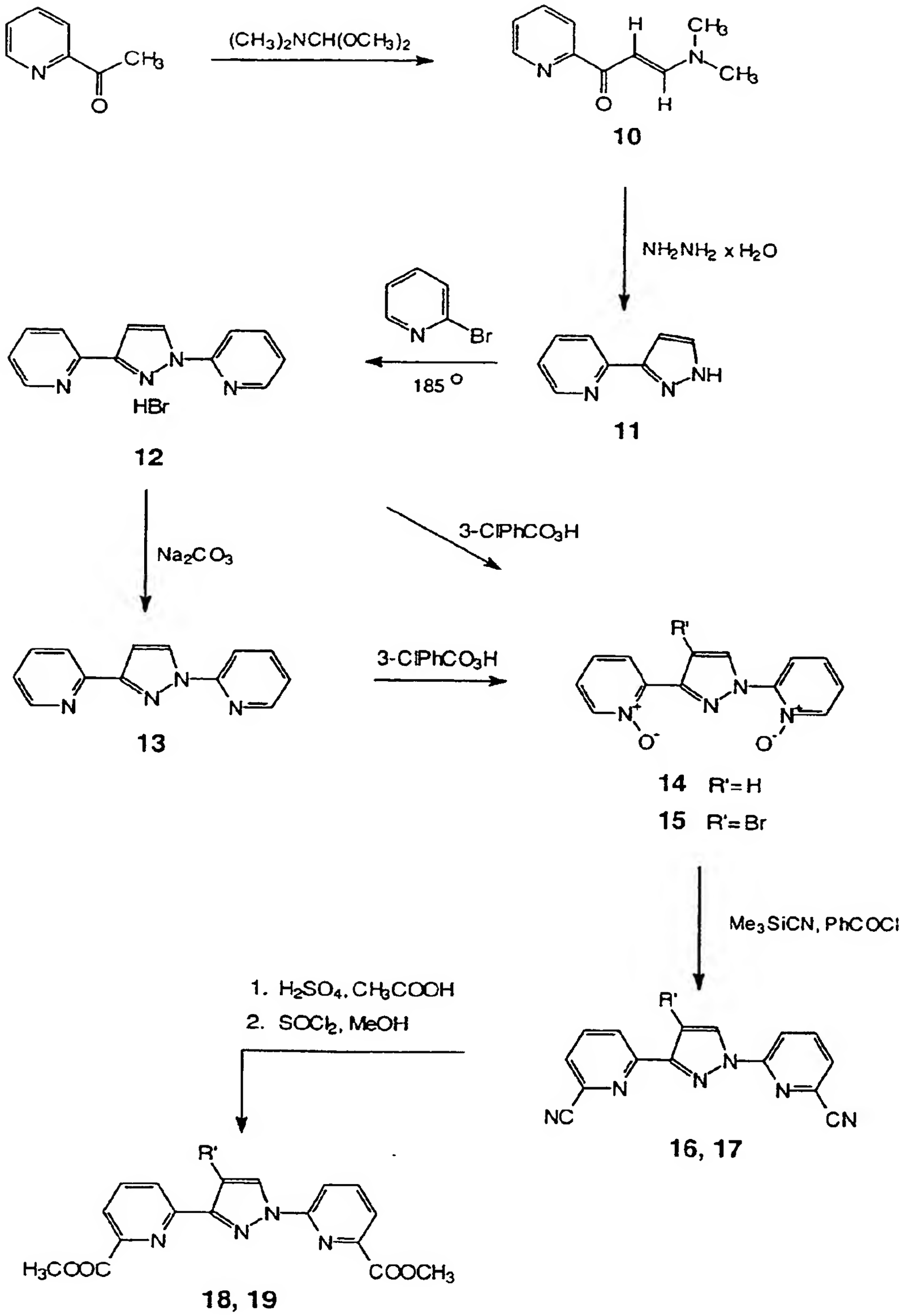

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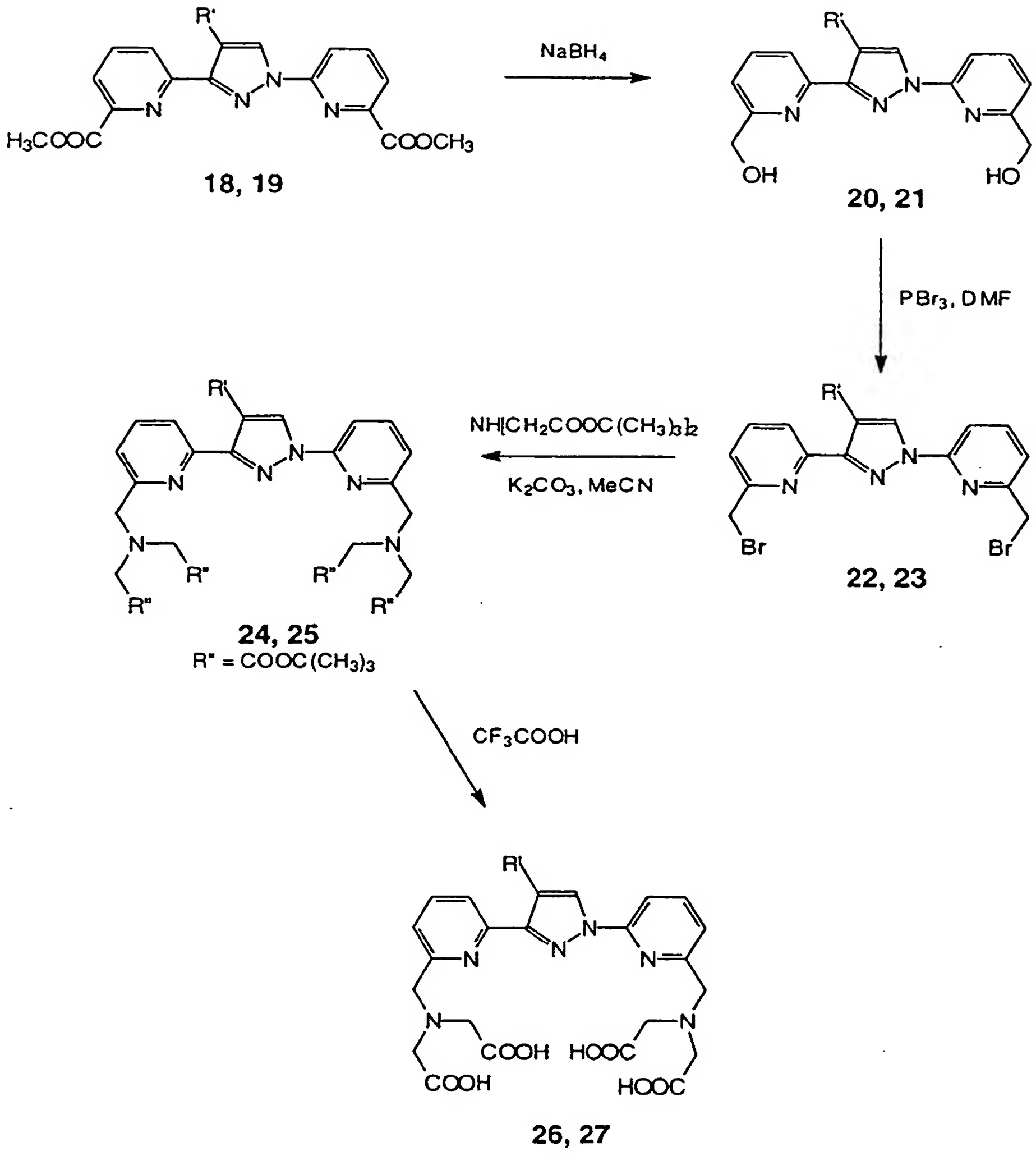

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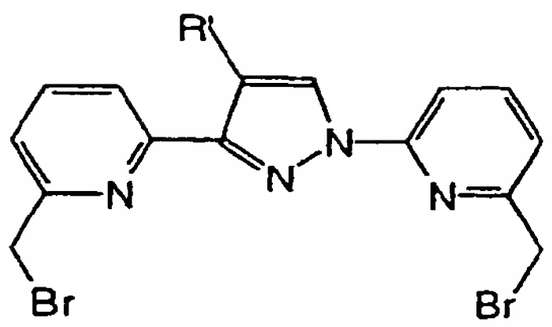
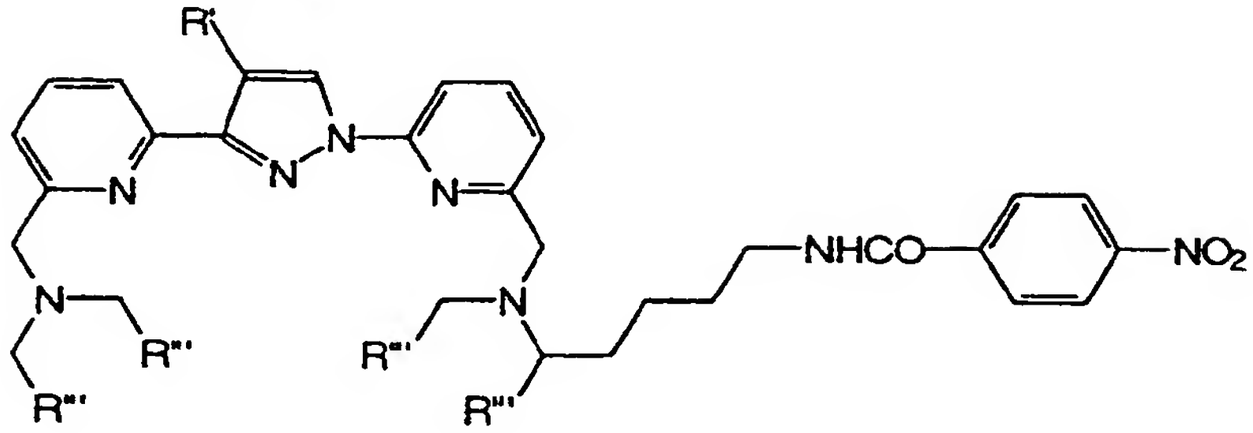


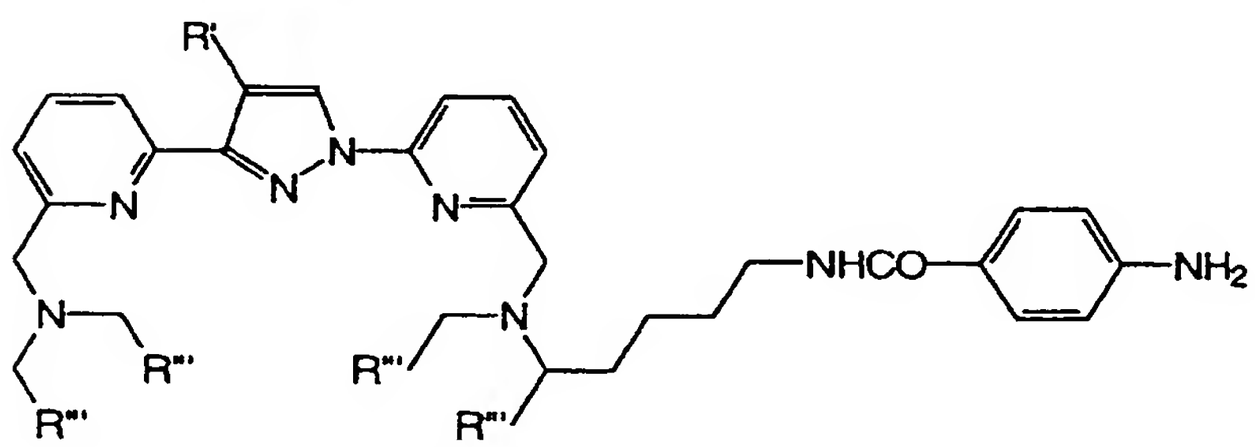
22, 23

4
$NH(CH_2COOCH_3)_2$
K_2CO_3, MeCN



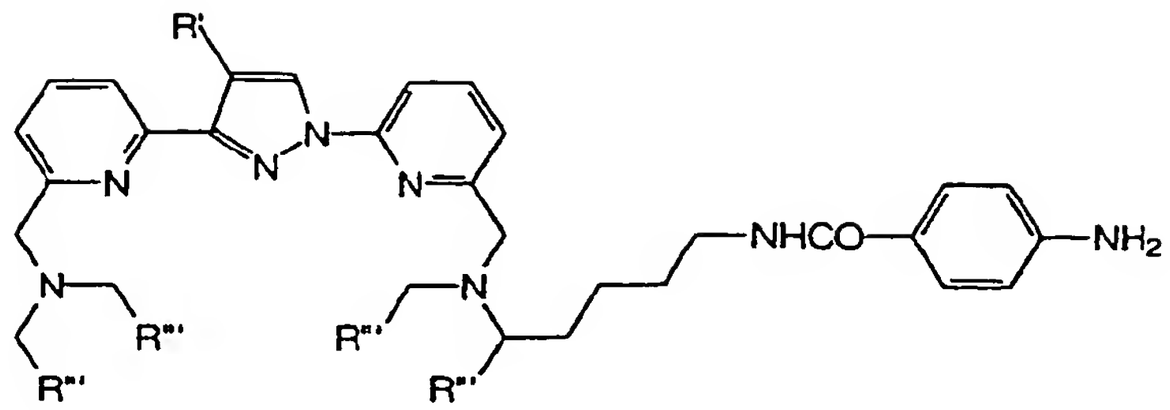
28, 29 $R''' = COOCH_3$

H_2, Pd/C



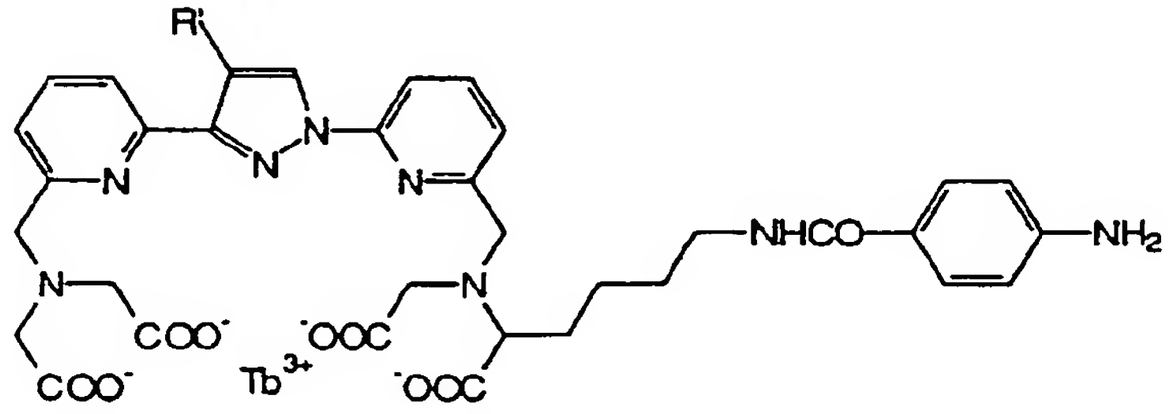
30, 31 one isomer shown

SCHEME 6.



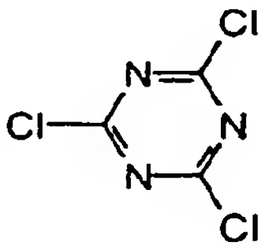
30, 31 one isomer shown

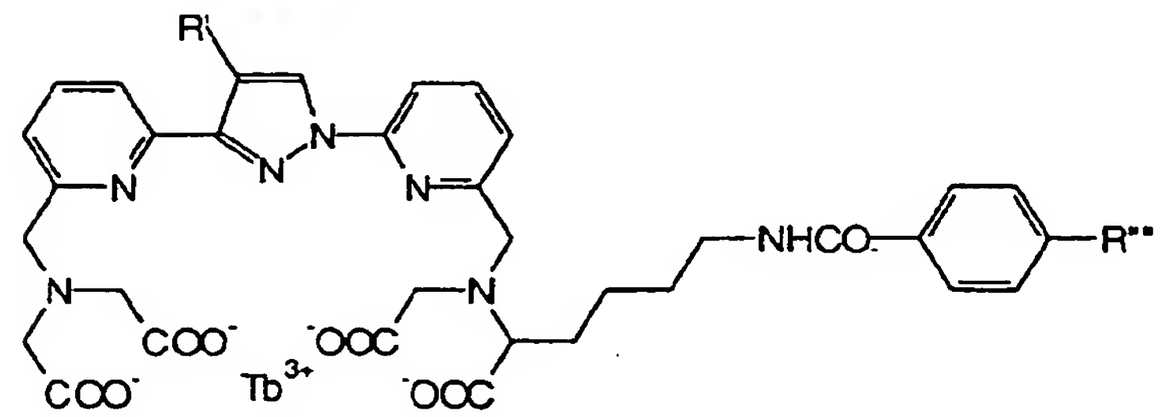
1. KOH, EtOH
2. $TbCl_3$



32, 33

$Cl_2C=S$ or

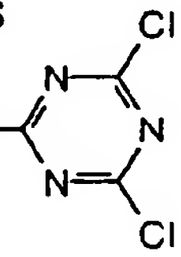




34 R' = H, R''' = NCS

35 R' = Br, R''' = NCS

36 R' = H, R''' =



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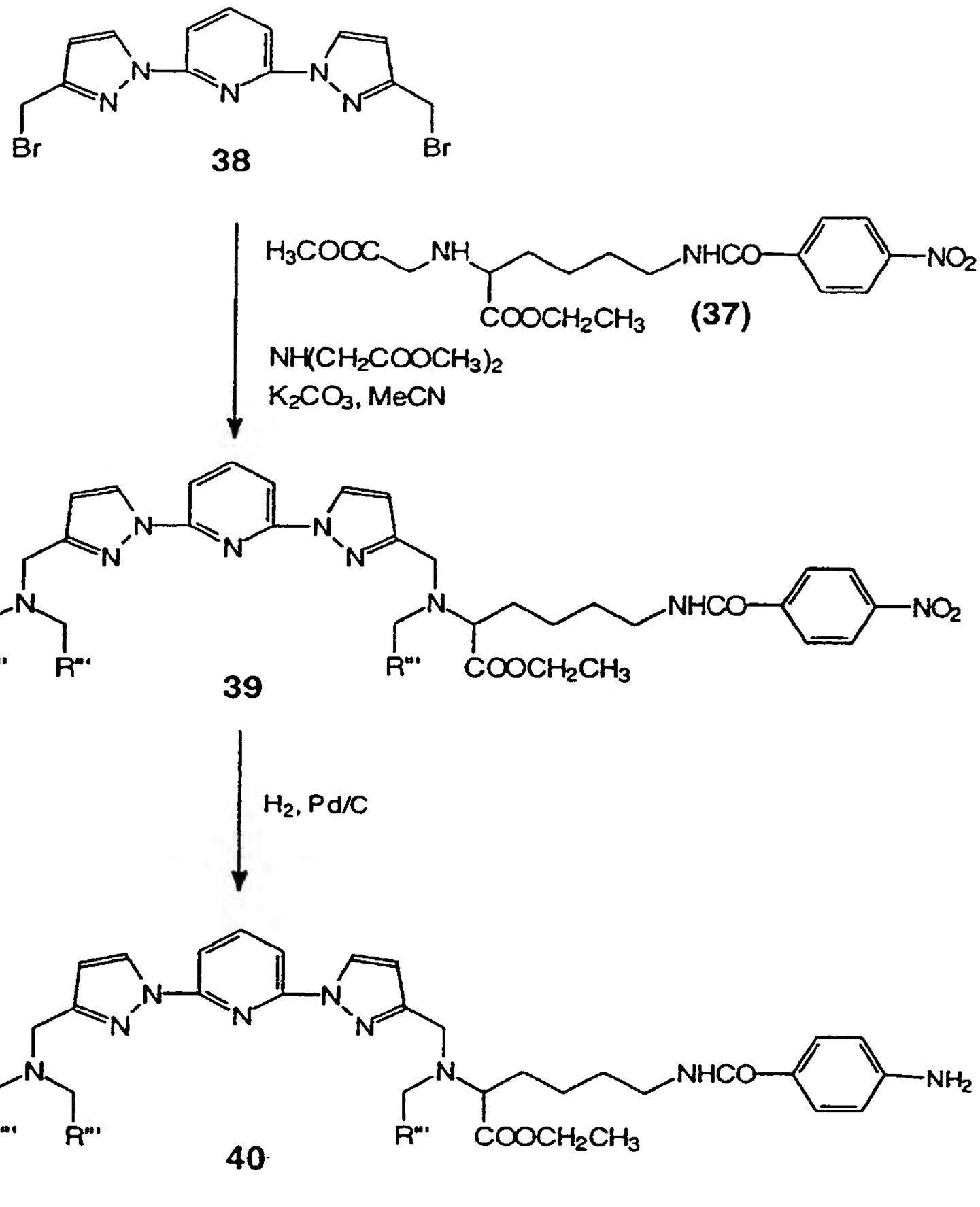

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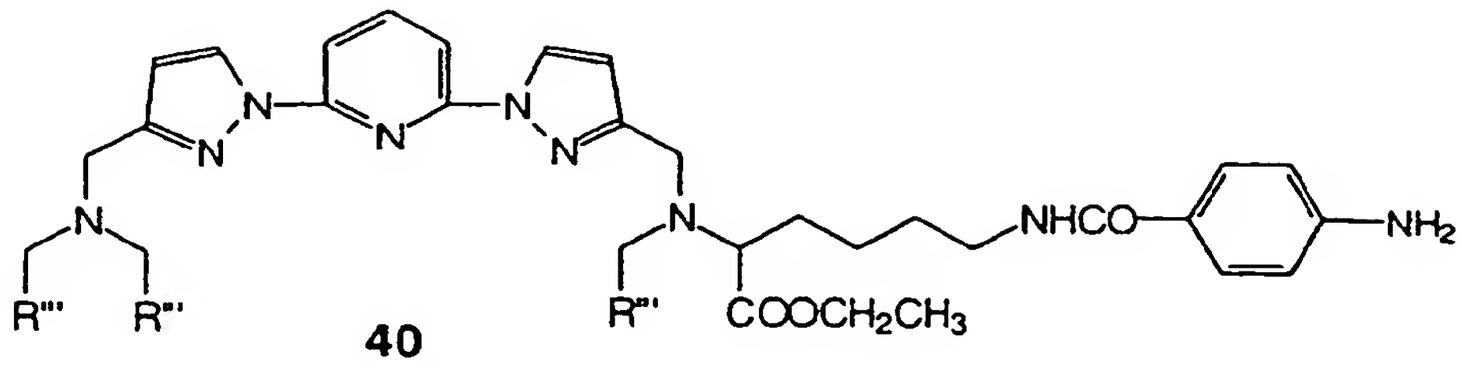
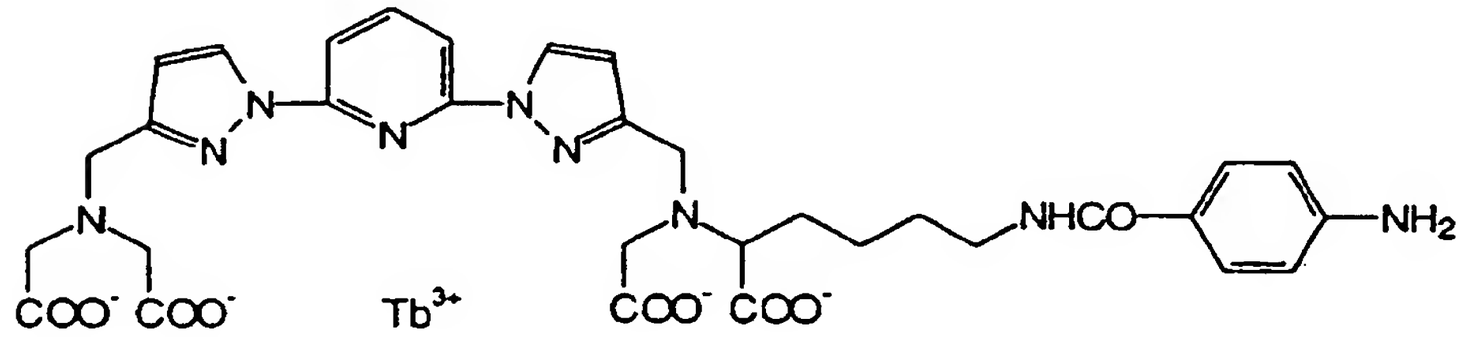


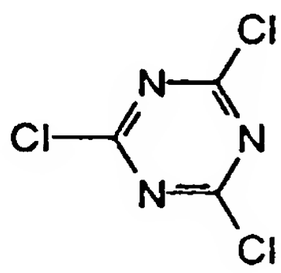
40

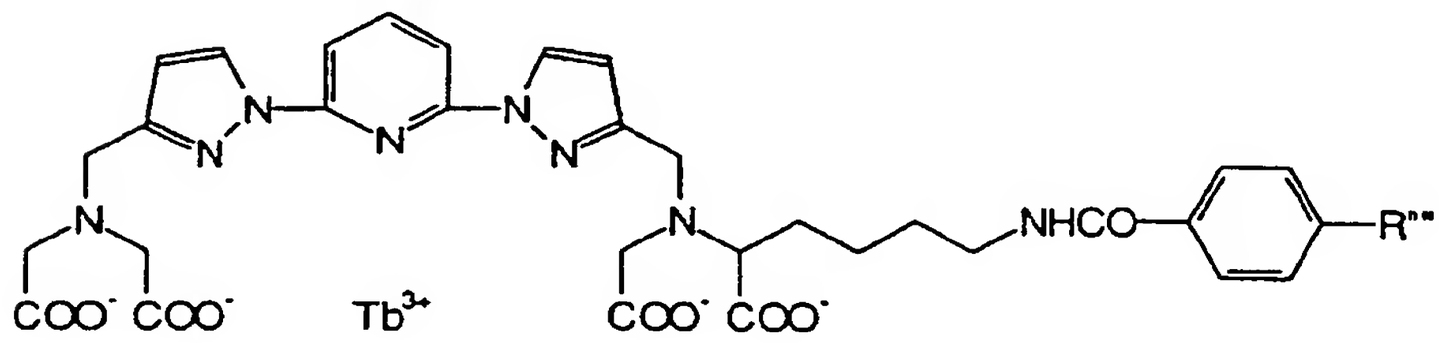
1. KOH, EtOH
2. $TbCl_3$



41

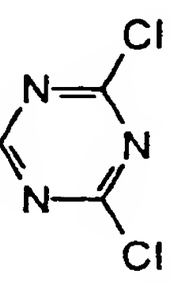
$Cl_2C=S$ or





42 R"" = NCS

43 R"" =



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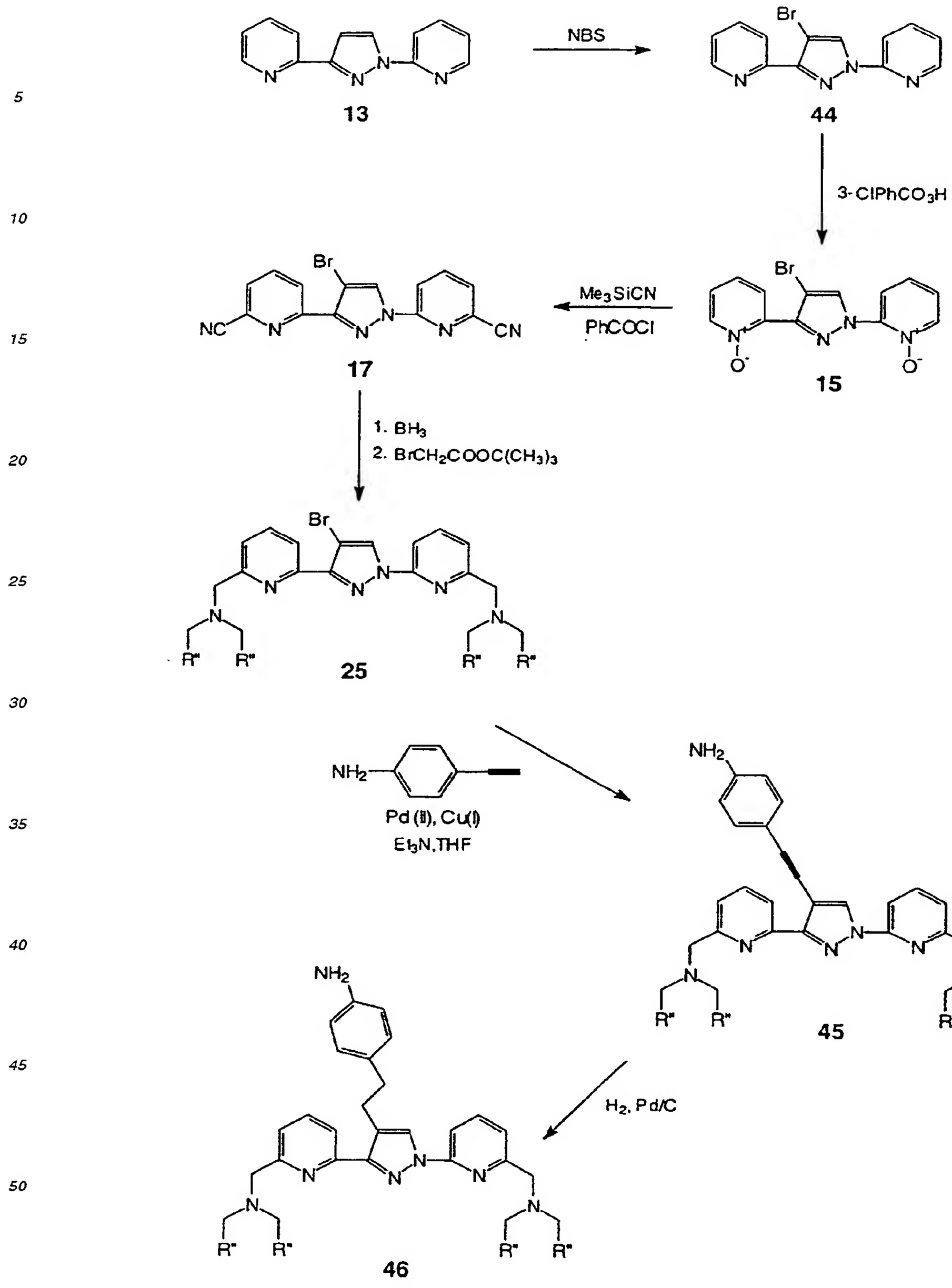

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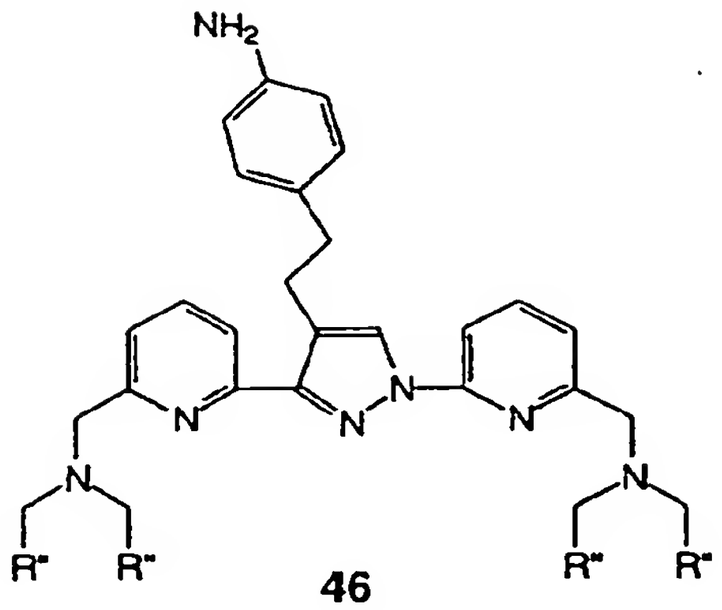
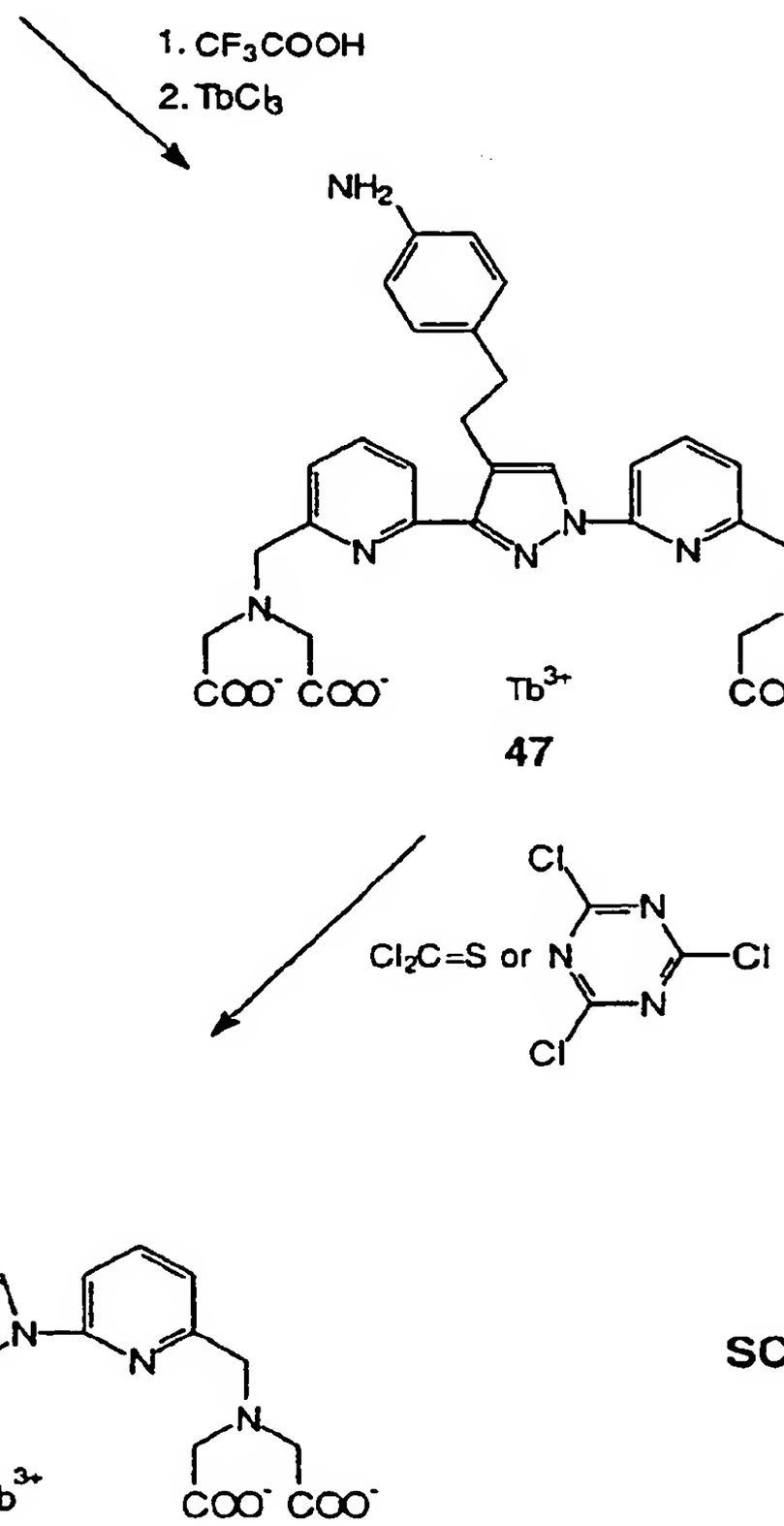


46



47

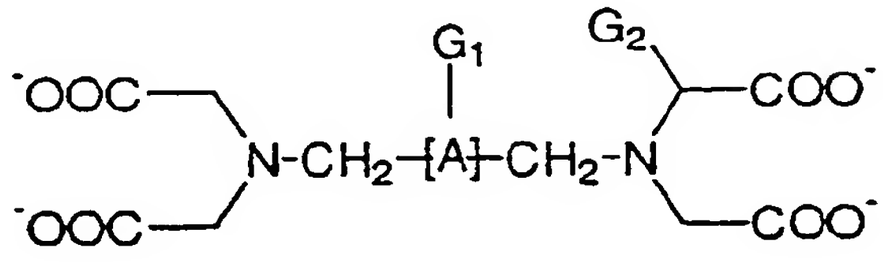
48 $R^{\prime\prime\prime\prime}$ = NCS

49 $R^{\prime\prime\prime\prime}$ =

SCHEME 11.

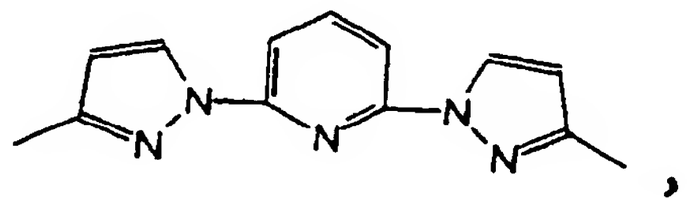
Claims

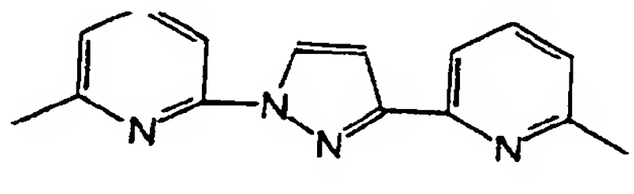
1. A detectable molecule comprising a biospecific binding reactant attached to a luminescent lanthanide chelate comprising a lanthanide ion and a chelating ligand of the formula



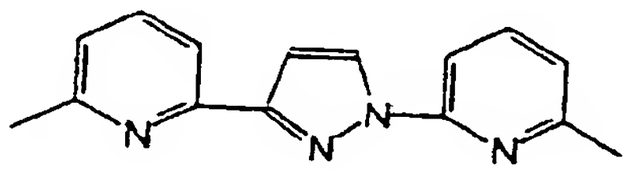
characterized in that

a) -A- is a bivalent aromatic structure selected from the group consisting of





and



b) one of the groups G_1 or G_2 is selected from a group consisting of hydrogen, chloro, bromo, iodo, cyano, phenyl, alkyl and alkoxy, with the proviso that alkyls contain 1-6 carbon atoms; and the other group G_1 or G_2 is a bridge which is not participating in the chelating process and which is formed of one to four moieties, each moiety being selected from the group consisting of phenylene, alkylene containing 1-8 carbon atoms, ethynediyl (-C≡C-), ether (-O-), thioether (-S-) and amide (-CO-NH- and -NH-CO-);

c) one of the two groups G_1 or G_2 is used for coupling to a biospecific binding reactant wherein the group G_1 or G_2 selected for this purpose is coupled to biospecific binding reactant via thiourea (-NH-CS-NH-), aminoacetamide (-NH-CO-CH_2-NH-), amide (-NH-CO- and -CO-NH-), aliphatic thioether (-S-), disulfide (-S-S-) or 6-substituted-1,3,5-triazine-2,4-diamine; and

d) the lanthanide ion is europium(III), terbium(III), dysprosium (III) or samarium (III).

2. The detectable molecule according to claim 1 **characterized** in that the biospecific binding reactant is selected from the group consisting of an antibody, an antigen, a receptor ligand, a specific binding protein, a DNA- or RNA-probe.

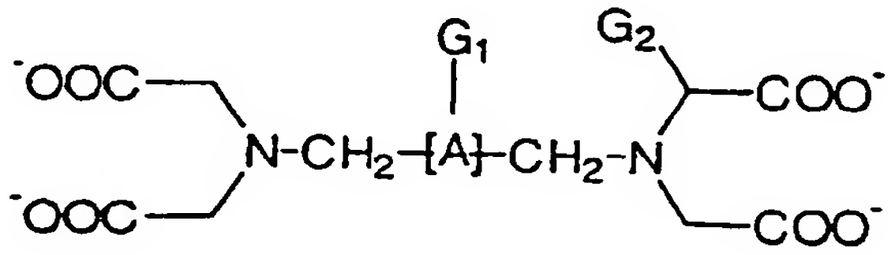
3. The detectable molecule according to claim 1 **characterized** in that the lanthanide chelate attached to a biospecific binding reactant is selected from the group consisting of

- 2- and 2"-[4-(4-isothiocyanatobenzamido)but-1-yl]-2,2',2",2"'-{[6,6'-(pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl]

bis(methylenenitrilo)}tetrakis(acetato) terbium(III) (**34**),

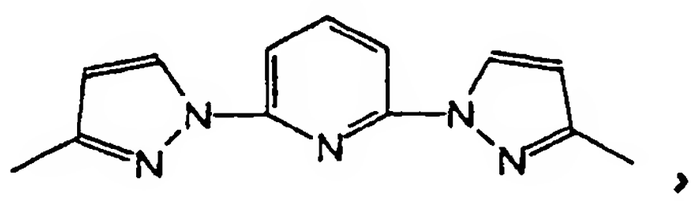
- 2- and 2"-{4-(4-isothiocyanatobenzamido)but-1-yl]-2,2',2",2"'-{[6,6'-(4"-bromopyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl]bis(methyl-enenitrilo)}tetrakis(acetato)terbium(III) (**35**),
- 2- and 2"-{4-{4-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]benzamido}but-I-yl}-2,2',2",2"'-{[6,6'-(pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl]bis(methyl-enenitrilo)}tetrakis(acetato)terbium(III) (**36**),
- 2-[4-(4-isothiocyanatobenzamido)but-1-yl]-2,2',2',2"-{[1,1'-(pyridine-2",6"-diyl)bis(pyrazole)-3,3'-diyl]-bis (methylenenitrilo)}tetrakis(acetato)terbium(III) (**42**),
- 2-{4-{4-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]benz-amido}but-1-yl}-2,2',2",2"'-{[1,1"-(pyridine-2" ,6"-diyl)bis (pyrazole)-3,3'-diyl]bis(methylenenitrilo))tetra-kis(acetato)terbium(III) (**43**),
- 2,2',2",2"'-{{6,6'-{4"-[2-(4-isothiocyanatophenyl)-ethyl]pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl}bis(methyl-enenitrilo)}tetrakis(acetato)terbium(III) (**48**), and
- 2,2',2",2"'-{{6,6'-{4"-{2-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]ethyl}pyrazole-1",3"-diyl)bis-(pyridine)-2,2'-diyl} bis(methylenenitrilo)}tetrakis-(acetato)terbium(III) (**49**).

4. A luminescent lanthanide chelate comprising a lanthanide ion and a chelating ligand of the formula



characterized in that

a) -A- is a bivalent aromatic structure selected from the group consisting of



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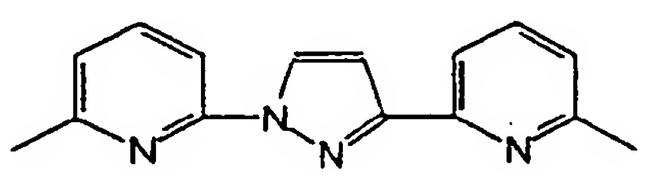
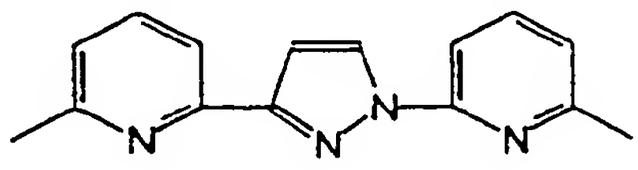


,

and



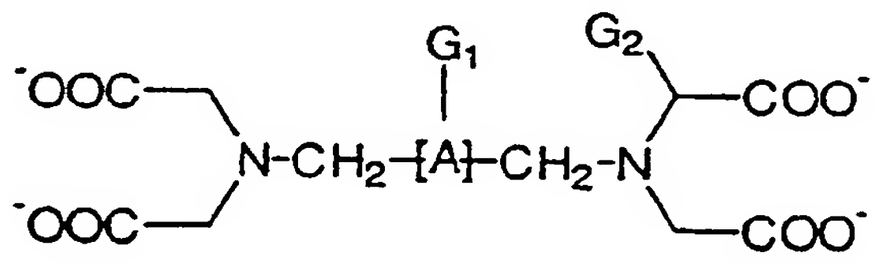
b) one of the groups G_1 or G_2 is selected from a group consisting of hydrogen, chloro, bromo, iodo, cyano, phenyl, alkyl and alkoxy, with the proviso that alkyls contain 1-6 carbon atoms; and the other group G_1 or G_2 is a substituent which is not participating in the chelating process and which is formed of one to four moieties, each moiety being selected from the group consisting of phenylene, alkylene containing 1-8 carbon atoms, ethynediyl (-C≡C-), ether (-O-), thioether (-S-) and amide (-CO-NH- and -NH-CO-) and additionally contains one moiety selected from a group containing hydroxy, nitro, amino, aminooxy, carboxyl, aldehyde or mercapto

groups or an activated form made from them such as isocyanato, isothiocyanato, diazonium, bromoacetamido, iodoacetamido, reactive esters, pyridyl-2-dithio or 6-substituted 4-chloro-1,3,5-triazon-2-ylamino;
c) one of the two groups G_1 or G_2 is used for coupling to a biospecific binding reactant; and
d) the lanthanide ion is europium(III), terbium(III), dysprosium (III) or samarium (III).

5. The lanthanide chelate according to claim 4 **characterized** in that the chelating ligand is selected from the group consisting of

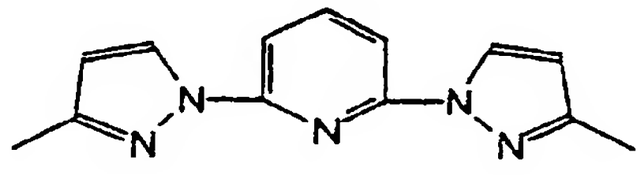
- 2- and 2"-[4-(4-isothiocyanatobenzamido)but-1-yl]-2,2',2",2"'-{[6,6'-(pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl] bis(methylenenitrilo)}tetrakis(acetato) terbium(III) (**34**),
- 2- and 2"-[4-(4-isothiocyanatobenzamido)but-1-yl]-2,2',2",2"'-{[6,6'-(4"-bromopyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl]bis(methylene-nitrilo)}tetrakis(acetato)terbium(III) (**35**),
- 2- and 2"-{4-{4-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]benzamido}but-1-yl)-2,2',2",2"'-{[6,6'-(pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl]bis(methyl-enenitrilo)}tetrakis(acetato)terbium(III) (**36**),
- 2-[4-(4-isothiocyanatobenzamido)but-1-yl]-2,2',2",2"'-{[1,1'-(pyridine-2",6"-diyl)bis(pyrazole)-3,3'-diyl]-bis (methylenenitrilo)}tetrakis(acetato)terbium(III) (**42**),
- 2-{4-{4-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]benz-amido}but-1-yl)-2,2',2",2"'-{[1,1"-(pyridine-2" ,6"-diyl)bis (pyrazole)-3,3'-diyl]bis(methylenenitrilo)}tetra-kis(acetato)terbium(III) (**43**),
- 2,2',2",2"'-{{6,6'-{4"-[2-(4-isothiocyanatophenyl)-ethyl]pyrazole-1",3"-diyl}bis(pyridine)-2,2'-diyl}bis(methyl-enenitrilo)}tetrakis(acetato)terbium(III) (48), and
- 2,2',2",2"'-{{6,6'-{4"-{2-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]ethyl}pyrazole-1",3"-diyl}bis-(pyridine)-2,2'-diyl} bis(methylenenitrilo)}tetrakis-(acetato)terbium(III) (**49**).

6. The use of a detectable molecule in biospecific binding assays, said detectable molecule comprising a biospecific binding reactant attached to a luminescent lanthanide chelate comprising a lanthanide ion and a chelating ligand of the formula



characterized in that

a) -A- is a bivalent aromatic structure selected from the group consisting of



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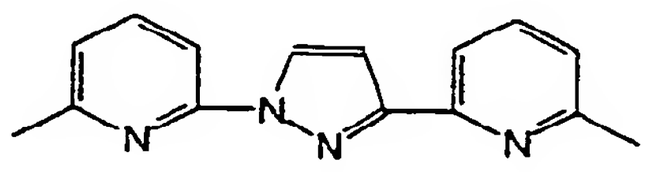
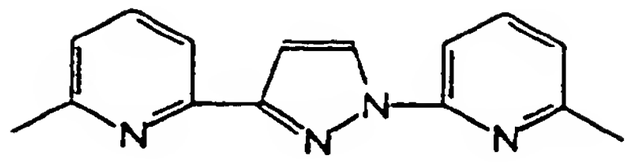


,

and



b) one of the groups G_1 or G2 is selected from a group consisting of hydrogen, chloro, bromo, iodo, cyano, phenyl, alkyl and alkoxy, with the proviso that alkyls contain 1-6 carbon atoms; and the other group G_1 or G_2 is a bridge which is not participating in the chelating process and which is formed of one to four moieties, each moiety being selected from the group consisting of phenylene, alkylene containing 1-8 carbon atoms, ethynediyl (-C≡C-), ether (-O-), thioether (-S-) and amide (-CO-NH- and -NH-CO-);
c) one of the two groups G_1 or G_2 is used for coupling to a biospecific binding reactant wherein the group G_1 or G_2 selected for this purpose is coupled to biospecific binding reactant via thiourea (-NH-CS-NH-), aminoacetamide (-NH-CO-CH_2-NH-), amide (-NH-CO- and -CO-NH-), aliphatic thioether (-S-), disulfide (-S-S-) or 6-substituted-1,3,5-triazine-2,4-diamine; and
d) the lanthanide ion is europium(III), terbium(III), dysprosium (III) or samarium (III).

7. The use according to claim 6 **characterized** in that the biospecific binding reactant is selected from the group consisting of an antibody, antigen, receptor ligand, a specific binding protein, a DNA- or RNA-probe.

8. The use according to claim 6 **characterized** in that the lanthanide chelate attached to a biospecific binding reactant is selected from the group consisting of

- 2- and 2"-[4-(4-isothiocyanatobenzamido)but-1-yl]-2,2',2",2"'-{[6,6'-(pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl] bis(methylenenitrilo)}tetrakis(acetato) terbium(III) (**34**),
- 2- and 2"-[4-(4-isothiocyanatobenzamido)but-1-yl]-2,2',2",2"'-{[6,6'-(4"-bromopyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl]bis(methylene-nitrilo)}tetrakis(acetato)terbium(III) (**35**),
- 2- and 2"-{4-{4-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]benzamido}but-1-yl)-2,2',2',2"-j[6,6'-(pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl]bis(methy-lenenitrilo)}tetrakis(acetato)terbium(III) (**36**),
- 2-[4-(4-isothiocyanatobenzamido)but-1-yl]-2,2',2',2"-{[1,1'-(pyridine-2",6"-diyl)bis(pyrazole)-3,3'-diyl]-bis (methylenenitrilo)}tetrakis(acetato)terbium(III) (**42**),
- 2-{4-{4-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]benz-amido}but-1-yl}-2,2',2",2"'-{[1,1'-(pyridine-2" ,6"-diyl)bis (pyrazole)-3,3'-diyl]bis(methylenenitrilo)}tetra-kis(acetato)terbium(III) (**43**),
- 2,2',2",2"'-{{6,6'-{4"-[2-(4-isothiocyanatophenyl)-ethyl]pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl}bis(methylenenitrilo)}tetrakis(acetato)terbium(III) (**48**), and
- 2,2',2",2"'-{{6,6'-{4"-{2-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]ethyl}pyrazole-1",3"-diyl)bis-(pyridine)-2,2'-diyl} bis(methylenenitrilo)}tetrakis-(acetato)terbium(III) (**49**).

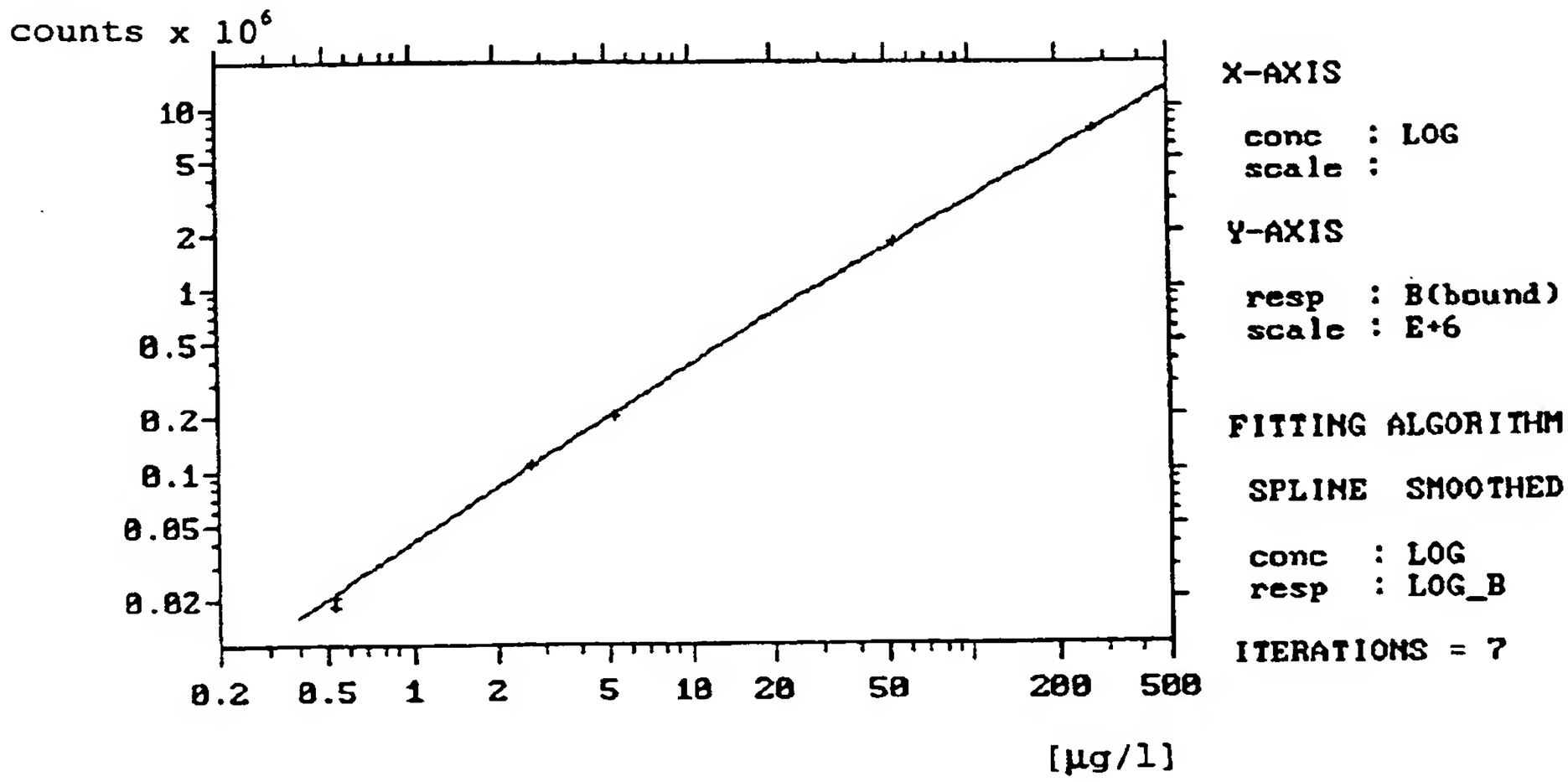


FIG. 1

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 96 66 0056

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,X	WO 93 11433 A (WALLAC OY) 10 June 1993 * page 65 - page 68; claim 1 * * page 69; claims 8,9 * -----	1-8	C07D401/14 G01N33/533

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

C07D

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	22 January 1997	Fink, D

CATEGORY OF CITED DOCUMENTS

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Y : particularly relevant if combined with another document of the same category
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L : document cited for other reasons

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